



Metabolite identification in *Ruditapes philippinarum* after exposure to an artificial sweetener (Acesulfame-K) and UV filter (4-MBC).

by
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Erasmus Mundus Joint Master in Water and Coastal Management. WACOMA
(10/08/2017)

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Author has been financially supported by Erasmus Mundus



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This Master Thesis was carried out in the Department of Physical Chemistry Faculty of University of Cádiz (UCA), as part of the UNESCO/UNITWIN/WiCoP activities at Cádiz, Spain, and in University of Bologna. The work was part of the project Integrated omic tools for the environmental risk assessment of emerging contaminants in marine species of commercial interest financed by Spanish Ministry of Science and Education CTM2015-70731-R.

STATEMENT

I hereby declare that this work has been carried out by me and the thesis has been composed by me and has not been submitted for any other degree or professional qualification.

This work is presented to obtain a masters degree in water and coastal management.

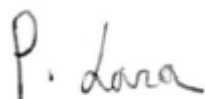
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HACEN CONSTAR:

Que esta Memoria, titulada “Metabolite identification in Ruditapes philippinarum after exposure to an artificial sweetener (Acesulfame-K) and UV filter (4-MBC)” presentada por D. Nieves del Rocio Colás-Ruiz resume su trabajo de Tesis de Máster y, considerando que reúne todos los requisitos legales, autorizan su presentación y defensa para optar al grado de Máster Erasmus Mundus in Water and Coastal Management (WACOMA).

10/08/2017



Dr. Pablo A. Lara Martín

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I dedicate this master thesis to my parents, my aunt and my brother.

ACKNOWLEDGEMENTS

I thank my guide **Prof Dr. Miriam Hampel** and my guide **Prof Dr. Pablo A. Lara-Martín**, of the department of Physical Chemistry from University of Cadiz, for their valuable support and advice. I acknowledge them for the opportunity of collaborating in such an interesting and innovative project, for the guidance through laboratory work and the writing process and also for the sympathy and the patience that they have always showed me during this learning process.

I acknowledge to **Emiliano Gómez** from the Central Services of Science and Technology and to **Marina Pintado** from the department of Physical Chemistry from University of Cadiz for their immeasurable support and help. Every discussion with them has been incredible enriching.

Many thanks to **Carmen López** for her managerial and administrative work and her support, to **Prof. Dra. Elena Fabbri** of University of Bologna, Italia, and to **Filomena Volpe** for their assistance in both, academically and administratively during my stay in Ravenna.

Gratitude to my master's colleagues that have taught me so much during these two years.

Recognitions to my very old friends, Maite, Julia, Laura, Borja, Carlos y Alberto, although they are far, I feel their encouragement closer than ever.

Last but not least many thanks to my family that always provide me unconditional support.

Outline of the thesis

Chapter 1: This chapter deals with a general introduction on organic pollutants in the environment and how the new analytical techniques have helped the scientific community to identify a new group of pollutants, the so called “emerging pollutants”. This chapter also describes the physicochemical characteristic of the two emerging pollutant selected for the present work, their occurrence and concentration found in the environment. It further addresses why the omics tools are essential to understand the possible adverse effect of these recent identified “emerging pollutants” on the organism and the types on omics tools.

Chapter 2: This chapter deals with the materials and methods for carrying out the present research work including exposition experiment, metabolites extraction method and metabolites identification methods as well as the post-acquisition data tools used.

Chapter 3: This chapter describes the obtained results after separation and identification of metabolites and after comparison of two extraction methods.

Chapter 4: This chapter describes the discussion of the obtained results offering an explanation on the behaviour of the used compounds. The results of this study are compared with previous relevant studies and the similarities and differences are discussed as well.

Chapter 5: conclusion.

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Acronyms

- OECD: Organisation for Economic Co-operation and Development
- POPs: Persistent Organic Pollutant
- EPs: Emerging Pollutant
- PPCPs: Pharmaceutical and Personal Care Products
- EQSD: Environmental Quality Standards Directive
- WFD: Water Framework Directive
- PCP: Personal Care Products
- WWTPs: Waste Water Treatment Plants
- PAHs: Polycyclic Aromatic Hydrocarbons
- PCBs: Polychlorinated biphenyl
- BP3: Benzophenone-3
- OC: Octocrylene
- EHMC: Ethylhexyl methoxycinnamate
- 4-MBC: 4-Methylbenzylidene Camphor
- NNS: Non-Nutritive sweeteners
- SAC: Saccharin
- CYC: Cyclamate
- ACE: Acesulfame-K
- SUC: Sucralose
- ALI: Alitame
- NEO: Neotame
- SCF: Scientific Committee on Food
- EU: European Union
- FDA: Food and Drug Administration
- EFSA: European Food Safety Authority
- LC-MS: Liquid Chromatography-Mass Spectrometry
- GC-MS: Gas Chromatography-Mass Spectrometry
- SPE: Solid Phase Extraction
- UPLC: Ultra Performance Liquid Chromatography
- QTOF: Quadropole Time Of Flight

- ESI: Electrospray Ionization
- APCI: Atmospheric Pressure Chemical Ionization
- SPLE: Selective Pressurized Liquid Extraction
- EICs: Extracted Ion Chromatograms
- SOD: Superoxide Dismutase
- Mn-SOD: Mn Superoxide Dismutase
- EC-SOD: ExtraCellular Superoxide Dismutase
- CAT: Catalase
- GPx: Glutathion Peroxidase
- GR: Glutathion Reductase
- GST: Glutathione-S-Transferase
- GSSG: Oxidized Glutathione
- GSH: Reduced Glutathione
- TrxP: Thioredoxin Peroxidase
- TrxR: Thioredoxin Reductase
- Trx(ox): Oxidized Thioredoxin
- Trx(red): Reduced Thioredoxin

ABSTRACT

In the last decades several pollutants have been discharged in to the marine ecosystem causing alarming effects that awoke the social concern, triggering the implementation of legislation. Nowadays several new pollutants are discharged to the marine ecosystem in small quantity but constantly. Due to the improvement of new analytical techniques these pollutants, the so called “emerging pollutants”, are been identified and its occurrence has been determined in the environment. However, very little is known about the possible adverse effect of these emerging pollutants in the organism. In this context the present work evaluates possible biotransformation products of two EPs by the Manila clam *Ruditapes philippinarum* in order to identify xenometabolites that could have negative impact in the functions of the organism.

UPLC-GC/ quadrupole-time-of-flight mass spectrometry (QTOFMS) with automated data analysis software (Metabolynx™) was applied to identify the metabolites of the UV filter 4-MBC and the artificial sweetener acesulfame-k in the tissue of the Manila clam. 6 different metabolites were identified for 4-MBC while no metabolites were identified for ACE-K, suggesting that the UV filter is accumulated and metabolised to facilitate its excretion while the artificial sweetener is almost not accumulated and excreted unchanged. These results are mainly due to the physicochemical properties of the compound.

The present study provides important information about the metabolism of 4-MBC and ACE-K which will be helpful for fully understanding the mechanism of action of these compounds. Furthermore, this work demonstrates the potential of the UPLC-GC/QTOFMS approach using Metabolynx™ software for fast and accurate identification of metabolites of EPs.

Hypothesis

Since Emerging Organic Pollutants are very similar to Persistence Organic Pollutants in terms of chemical structure and environmental behavior, they have the ability to persist and bioaccumulate in the marine environment in similar or even higher levels than other regulated substances such as PCBs or organochlorine pesticides, which production and use is restricted due to their very well known environmental toxicity.

Aim of the work

The main aim of this research work is to study the metabolization process of two selected emerging pollutants (UV filter and artificial sweetener) by a bivalve specie very well known for its commercial interest, the Manila clam *Ruditapes philippinarum*.

The development of this overall objective involves the following specific objectives:

- Optimize the existing methodology for the extraction and analysis of metabolites.
- Comparison of two different analytic techniques in emerging organic pollutants in order to determine their suitability in the analysis of biotransformation products.
- Identification of biotransformation products of the selected EP by the Manila clam.
- Determination of the chemical structure of the identified byproducts as well as the biotransformation pathway.

Chapter 1

General Introduction

1. Introduction

Over recent decades, the development of multiple human activities (industry, transport, agriculture) has led to the increase in the standard of living, triggering the rise of demand and consumption for goods, especially in developed countries. In order to satisfy the market requirements, many different contaminants have been released into the environment due to the industry process and consumption of those goods, producing effects in the environment such as, pollution in the air (with CO₂, NO_x, SO₂, greenhouse gasses, particulate matters), water (with synthetic organic chemicals, nutrients, oil spills) and soil (due to the disposal of hazardous waste, spreading of pesticides) (Gavrilescu, 2010; Gavrilescu et al., 2015; OECD, 2015)

Since the first negative effects of the massive discharge of organic synthetic chemicals into the aquatic compartment were noticed in ecosystems and human health (around 60s-70s) (e.g., Sumpter, Jenkins, & Dodd, 1978), most of the environmental monitoring efforts have been done in Persistent Organic Pollutants (POPs) and therefore the presence, bioaccumulation and toxicity of these pollutants is very well-known. This information generated an important public concern that triggered the development of environmental policies and legislation to regulate synthetic organic chemical discharges. (Jones & de Voogt, 1999; Valle C, et al., 2016)

As a result of these environmental monitoring programmes, the Stockholm Convention on Persistent Organic Pollutants, was adopted on 22 May 2001 and entered into force on 17 May 2004 with the aim of eliminating or restricting the production and use of POPs (Ahlgren, 2014).

POPs, as well as other well studied compounds such as heavy metals are also known as “existing contaminants” (Edwards and Kjellerup, 2013), whereas there is an ever increasing number of newly developed chemicals which are called “emerging pollutants”. Although the adverse consequences of these synthetic organic pollutants are very well known, there is a lack of knowledge about these “emerging pollutants” (EPs). Recently, emerging pollutants that involve substances such as pharmaceuticals and personal care products (PPCPs), food additives and pesticides have increasingly gained attention due to their ubiquitous occurrence in

the aquatic environment and their potential to cause undesirable ecological effects (Birch et al., 2015; Tran, Hu, & Ong, 2013)

EPs are defined as synthetic or naturally occurring chemicals that are not commonly monitored in the environment but which have the potential to enter the environment and cause known or suspected adverse ecological and (or) human health effects. In some cases, release of emerging pollutants to the environment has likely occurred for a long time, but may not have been recognized until new detection methods or techniques were developed. In other cases, synthesis of new chemicals or changes in use and disposal of existing chemicals can create new sources of emerging pollutants (Geissen et al., 2015). Although there is no specific legislation regarding the monitoring of EPs, in the European Union, a dynamic watch list of EPs is elaborated from national monitoring programs. This mechanism, supported by Directive 2008/105/EC (the Environmental Quality Standards Directive, EQSD) has the aim to identify priority substances for regulation under the Water Framework Directive (WFD 2000) (Carvalho et al., 2015).

EPs are categorized into more than 20 classes related to their origin (fig. 1.1). The prominent classes are: pharmaceuticals (urban, stock farming), pesticides (agriculture), disinfection by-products (urban, industry), wood preservation and industrial chemicals (industry) and personal care products (Geissen et al., 2015).

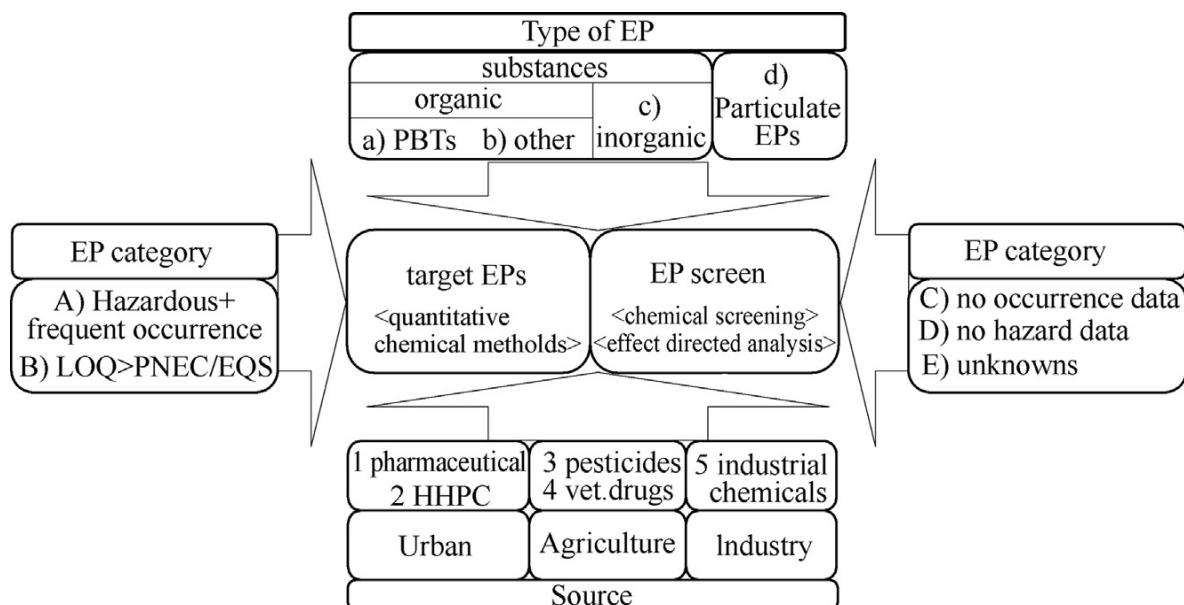


Fig. 1.1: Different types of Emerging pollutants according to their source (Geissen et al., 2015)

One of the main groups of EPs are **personal care products (PCPs)**, substances that are widely consumed by the society, indispensable for modern society (e.g., surfactants, fragrances, UV filters, antimicrobials, etc.) and continuously introduced in the environment mainly through the effluents of wastewater treatment plants (WWTPs).

There are two pathways by which these PCPs enter the aquatic environment have been identified (fig. 1.2):

- **Directly**, as consequence of recreational water activities (e.g., bathing and swimming)
- **Indirectly**, mainly via WWTPs, as result of the use of cosmetics, showering, washing, rubbing off after dermal application and excretion (Díaz-Cruz and Barceló, 2009).

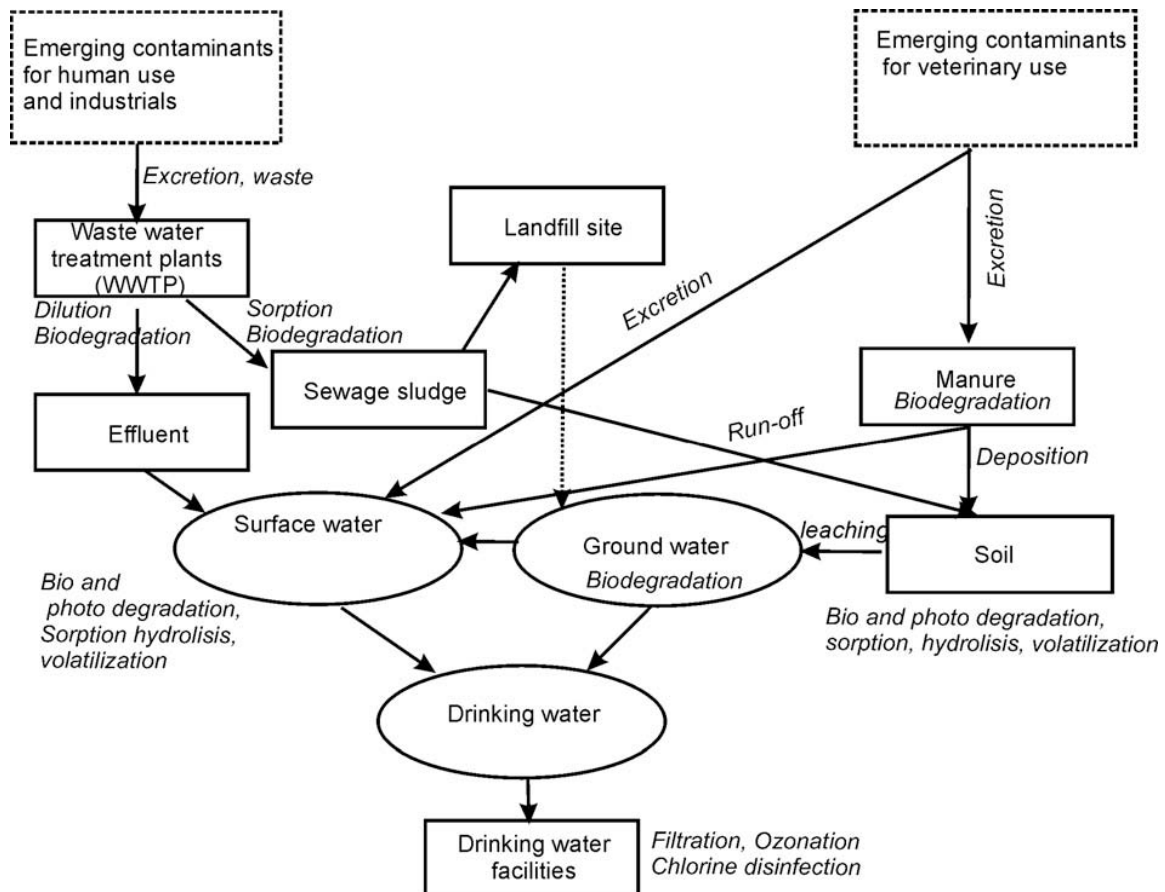


Fig. 1.2: Distribution of synthetic chemicals and main transformations in the environment and the technosphere.(Farré et al., 2008)

Within **food additives**, artificial sweeteners are recently recognized as a new class of emerging environmental pollutants. They are used worldwide as sugar substitutes in remarkable amounts in food, beverages, and also in drugs and sanitary products, such as mouthwashes. This type of emerging pollutant are highly specific to wastewater, as they are released to the environment after human consumption (Lange et al., 2012).

Currently, detection of EPs at very low concentrations due to the advances in analytical techniques has revealed a worldwide issue. They have been found in surface waters (e.g., lakes and rivers), wastewater, drinking water, soil, sludge, and fauna (Díaz-Cruz and Barceló, 2009; Díaz-Garduño et al., 2017). In fact, EPs have been detected in traditionally non polluted areas or areas far from the main source of contamination, such as the Polar Regions (Esteban et al., 2016; González-Alonso et al., 2017).

Although studies and reviews can be found in the literature on sources, occurrence, environmental behaviour and fate of emerging contaminants, the potential risk for the receptors continues to be an essential subject for advanced research (Gavrilescu, 2010; Gavrilescu et al., 2015; Geissen et al., 2015). As an example, several studies have been conducted in Cadiz Bay (area of study of the present work) about distribution and occurrences of EPs such as, PAHs, PCBs, several types of pesticides (e.g., organochlorines, organophosphorus, and pyrethroids), organophosphate flame retardants, antimicrobials, nonylphenol, fragrances, UV filters, pharmaceutical products, Synthetic surfactants (Baena-Nogueras et al. 2016; Corada-Fernández et al. 2015; Pintado-Herrera, et al., 2014; Pintado-Herrera et al., 2017; Pintado-Herrer et al., 2016). However no previous studies have been conducted on the possible adverse effect of these EPs in the organism of the area. In this context the present work analyses the possible biotransformation products of two EPs by the Manila clam *Ruditapes philippinarum* in order to identify degradation products or metabolites that could have possible adverse effects in the functions of the organism.

1.1. Sunscreen and UV filters.

Although life in earth would be impossible without solar radiation (Nohynek & Schaefer, 2001) since hominids lost their covering of hair and exposed their skin to sunlight, around 1. 2 million years ago (Rogers et al., 2004) the need to protect themselves from excessive radiation arose. Ancient Egyptians were the first to use a kind of sunscreen made of olive oil, and this was also the case in Greece and Rome. However, it was at the end of the nineteenth century when the first scientific research into sunscreen protection was reported. (Sánchez-Quiles and Tovar-Sánchez, 2015).

UV filters (UV-Fs) belong to the largest group of PCP and are used extensively in a variety of personal care products such as sunscreens, foundations, lipstick, eye liner and shadows, as well as in many industrial materials such as plastics, adhesives and rubber. UV filters are able to absorb UV radiation and thereby protect human skin from direct exposure to the deleterious wavelengths of sunlight and protect products against photo-degradation (Gago-Ferrero et al., 2015; Giokas et al., 2007).

There are two basic types of UV filters, organic (also known as physical) and inorganic (known as chemicals) compounds. The organic UV filters are responsible of the abortion of UV light, mainly UVB. The inorganic UV filters (TiO_2 , ZnO), which are considered to be biologically inert and nontoxic substances also reflect and scatter UV light. Generally, both types of UV filters give good protection against UVB (280-315 nm), and some also offer protection against UVA (315-400 nm) radiation (Balmer, Buser, Müller, & Poiger, 2005; Nohynek & Schaefer, 2001; Shaath, 2010; Silvia Díaz-Cruz, Llorca, Barceló, & Barceló, 2008)

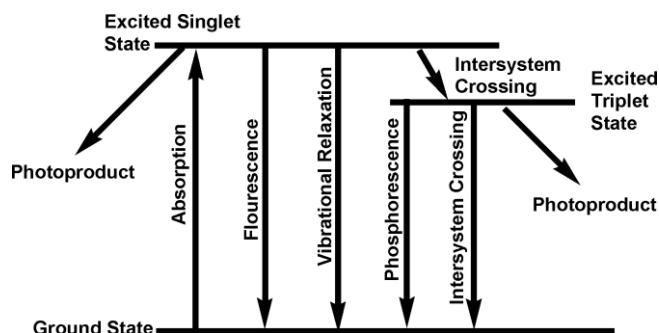


Fig. 1.3: Energy release pathways (Shaath, 2010)

These compounds usually possess single or multiple aromatic structures, sometimes conjugated with carbon-carbon double bonds and/or carbonyl moieties, able to attenuate the transmission of energetic solar photons that reach the surface of

the Earth. More specifically, UV filters absorb photons and rapidly return them to

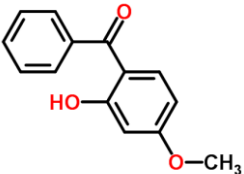
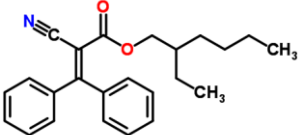
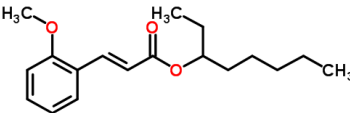
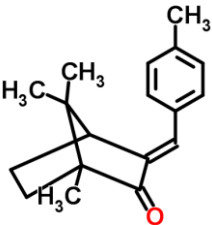
ground state by thermally emitting the energy through a series of vibrational relaxation (fig. 1.3). Then, if no degradation of the UV filters is produced, they can absorb additional photons and repeat the process, thereby protecting the skin from UV radiation (Giokas et al., 2007; Shaath, 2010).

There are currently 27 UV filters permitted by the EU Cosmetics Directive for commercial use, of which 26 are organic UV filters and only one (TiO_2) is inorganic (Giokas et al., 2007). Table 1 shows the most relevant physicochemical properties of the most used UV filters (Benedé, et al. 2014; Giokas et al., 2007)

Although for many years most of the research around the use of UV-filters has been focused on developing more effective sunscreens in order to increase market demand (Nohynek & Schaefer, 2001; Giokas et al., 2007), there is a recent concern about UV filters as emerging contaminants and their implications into the coastal marine system (Gago-Ferrero et al., 2015; Sánchez-Quiles and Tovar-Sánchez, 2015)

Coastal tourism is one of the main sources of UV filter discharge in near shore waters (Tovar-Sánchez et al., 2013) and due to its persistence and low degradability, they have been found in different levels of the food chain even at the human level. In a study, Schlumpf et al. (2010) found residues of six different types of UV filters in 85.2% of the samples of human breast milk analysed, with the risk of transfer to the newborn infant as a result. Additionally these compounds have been proved to bioaccumulate in invertebrate and fish and have endocrine disruptive effect (Benedé et al., 2014b; Díaz-Cruz et al., 2008; Fent et al., 2010, 2008; Gago-Ferrero et al., 2015) For instance, Klann et al. (2005) found that 4-MBC has the potential to change physiological and developmental processes mediated by activating the estrogens receptors and inducing gene regulation in the African clawed frog at micro molar concentrations.

Table 1.1: Physic-chemical properties of the most used UV filters (Source: ChemSpider. <http://www.chemspider.com/>)

Compound	Chemical structure	Formula	Average mass (Da)	Water solubility at 25°C (mg L ⁻¹)	Log K _{ow}
Benzophenone-3 / Oxybenzone (BP3)		C ₁₄ H ₁₂ O ₃	228.243	68.56	3.52
Octocrylene (OC)		C ₂₄ H ₂₇ NO ₂	361.477	0.13	6.88
Ethylhexyl methoxycinnamate (EHMC)		C ₁₈ H ₂₆ O ₃	290.397	0.15	5.80
4-Methylbenzylidene Camphor (4-MBC)		C ₁₈ H ₂₂ O	254.367	0.57	5.92

1.1.1. 4-Methylbenzylidene Camphor (“4-MBC”)

4-MBC is an organic camphor derivative that is used in the cosmetic industry for its ability to protect the skin against UV, specifically UV-B radiation (Sakkas et al., 2009). Currently, is one of the most commonly employed UV filters (Badia-Fabregat et al., 2012) and is listed in the Cosmetics Directive 76/768/EEC as UV-filter which cosmetic products may contain (ref. no. 18 in Annex VII Cosmetics Directive 76/768/EEC) (SCCP, 2008).

4-MBC has a high octanol-water partition coefficient (K_{ow}) (table 1) that makes it very hydrophilic and leads to bioaccumulation in fish tissues. It seems to be chemically stable and not readily biodegradable (Sakkas et al., 2009)

It is found both in WWTPs and the environment but its concentration levels reported fluctuate significantly as a function of sample location, size of the studied system (e.g., lakes and swimming pools), type of recreational activities and sampling period as it is especially during summer when people use sunscreens for protection from UV radiation (Klann et al., 2005; Sakkas et al., 2009). Thus, in the aqueous environment concentrations between 2 to 82 ng/L have been found (Díaz-Cruz et al., 2008). On the other hand analysis of domestic sewage in various WWTPs across Switzerland revealed concentrations in the range 560-6500 ng L⁻¹ for 4-MBC (Giokas et al., 2007) while in south Australia Liu et al. (2011) detected levels up to 250 ng g⁻¹ in the sludge of WWTP. In different areas of the province of Cadiz (Puerto Real, Jerez, Rio San Pedro) 4-MBC has been found in concentrations between 46 to 49 (ng L⁻¹) in a river, sea and ground water and in a WWTP effluent, respectively. The concentrations in the studied area are less than the average, mainly due to the sampling period which was in fall instead of summer when UV filters are mainly used (Pintado-Herrera et al., 2014). However, and as mentioned before, there is no information available on the potential effects that the exposure to these kind of compounds could have on the organisms living within these ecosystems.

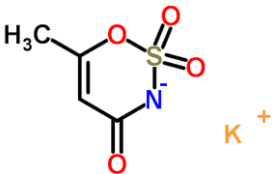
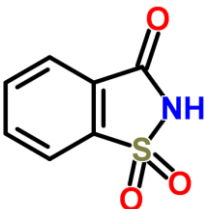
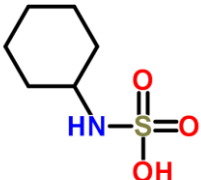
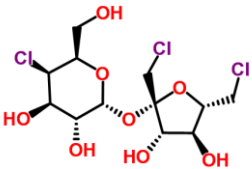
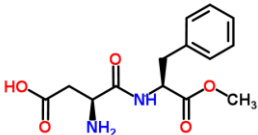
1.2. Artificial sweeteners.

Sweeteners are food additives, which are commonly used as sugar substitutes in food, beverage, confectionery, pharmaceutical and sanitary products that provide the sensation of sweetness (Gan et al., 2013; Ordóñez et al., 2012; Zygler et al., 2009).

The 'first generation' sweeteners were already introduced in the 1950s such as saccharin (SAC, code for food contents E 954), cyclamate (CYC, E 952) and aspartame (ASP, E 951), followed by the 'new generation' sweeteners acesulfame (ACE, E 950), sucralose (SUC, E 955), alitame (ALI, E 956) and neotame (NEO, E 961) (Berset and Ochsenbein, 2012).

They can be divided in two main groups: caloric, or nutritive, and non-caloric or non-nutritive sweeteners (NNS). Nutritive sweeteners are carbohydrates or their derivatives such as glucose, fructose and maltose. Non-nutritive sweeteners do not belong to any particular chemical group and they are usually known as artificial sweeteners. After ingestion, most of the NNS pass through the human metabolism largely unaffected, are quantitatively excreted via urine and faeces, and thus reach the environment associated with domestic wastewater (Buerge et al., 2009). Artificial sweeteners are steadily increasing in importance with increased public awareness of diabetes and its special dietary requirements, and with more consumers becoming concerned about obesity and dental caries. Artificial sweeteners are widely used all over the world, and some of them have a long history. For example, saccharin was invented nearly 100 years ago. Artificial sweeteners are also cheaper than natural sugar and can reduce the cost for some foods and beverages. (Ferrer and Thurman, 2010)

Table 1.2: Physic-chemical properties of the most used UV filters (Source: Lange et al., 2012; ChemSpider. <http://www.chemspider.com/>)

Compound	Chemical structure	Formula	Average mass (Da)	Water solubility at 20°C (g L ⁻¹)	Log k _{ow}
Acesulfame-K (ACE)		C ₄ H ₄ KNO ₄ S	201.242	270	-1.33
Saccharin (SAC)		C ₇ H ₅ NO ₃ S	183.184	4	0.91
Cyclamic acid (CYD)		C ₆ H ₁₃ NO ₃ S	179.237	1000	-1.61
Sucralose (SUC)		C ₁₂ H ₁₉ Cl ₃ O ₈	397.634	283	0.229
Aspartame		C ₁₄ H ₁₈ N ₂ O ₅	294.303	4	0.07

Although a very large number of artificial sweeteners are known, only few of them are allowed to be used in modern food industry. The list of authorized artificial sweeteners varies from country to country. For instance there are six artificial high-intensity sweeteners authorized for use in the European Union (EU) acesulfame, aspartame, cyclamate, saccharin, sucralose and neohesperidin dihydrochalcone (Ordóñez et al., 2012). Conversely, in the USA or Japan, the corresponding list excludes cyclamates and neohesperidine dihydrochalcone, while neotame is included (Bergamo et al., 2011; Zygler et al., 2009)

In January 2004, the EU amended its Sweeteners Directive permitting the use of several sweeteners in nutrition products. However, scientific research has shown that some artificial sweeteners can cause tumours in certain animals (Ferrer and Thurman, 2010).

Although most of the toxicological effects of NNS on humans have been discounted or dismissed by the European Food Safety Agency (EFSA) there are still suspicions in the scientific community about the possible adverse health effects of the mentioned sweeteners due to diverse studies (Wasik et al., 2007; Zygler et al., 2009, 2009). For instance some experiments have associated saccharin with bladder cancer when fed at high doses to rats, and aspartame with cancer, lymphomas and leukaemia also in rats. Additionally, the consumption of aspartame is not recommended in people with phenylketonuria, because excess intake of phenylalanine (one of the aspartame's metabolites) can lead to brain damage (Zygler et al., 2009).

Recently, the ecotoxicology of artificial sweeteners on aquatic and terrestrial organisms has drawn much attention, specially the consequences of low-dose exposure in relation with their persistence and way of discharge into the environment (Gan et al., 2013). Especially since 2009 the number of scientific articles on the environmental occurrence, fate, and ecotoxicological effects of artificial sweeteners has been rapidly increasing (Lange et al., 2012)

After decades using artificial sweeteners, recent studies have documented their widespread environmental occurrence; some of them (ACE, CYC, SAC and SUC)

have been detected in municipal wastewater and in ground and surface water (Zygler et al., 2009).

In particular, ACE was found in the aquatic environment in much higher concentrations than most personal care product ingredients and other anthropogenic organic chemicals. Whereas others artificial sweeteners such as CYC and SAC are usually degraded by more than 90% during wastewater treatments, ACE is not eliminated in WWTPs and is also quite persistent in surface waters (Buerge et al., 2009; Lange et al., 2012; Ordóñez et al., 2012). For instance Buerge et al. (2009) detected ACE in untreated and treated wastewater (up to $46 \mu\text{g L}^{-1}$), and even in several tap water samples (up to $2.6 \mu\text{g L}^{-1}$) from Switzerland. In Europe the average of WWTP effluent concentration of ACE was $76.1 \mu\text{g L}^{-1}$, with a maximum concentration detected of 2.5 mg L^{-1} (Loos et al., 2013). In areas of Canada (i.e. city of Barrier, Ontario) ACE was found in groundwater samples at concentrations up to $33.6 \mu\text{g L}^{-1}$ (Van Stempvoort et al., 2011).

1.2.1. Acesulfame-k

Acesulfame-K (potassium) (5,6-dimethyl-1,2,3-oxathiazine-4(3H)-one-2,2-dioxide) was accidentally discovered in 1967 by chemists Karl Clauss and Harald Jensen. They noticed a sweet taste when they licked their finger while working in the laboratory (Cantarelli et al., 2009; Nabors, 2004; Pierini et al., 2013). ACE is a white, crystalline powder. The crystals are monoclinic.

ACE was approved in the United States in 1988 for specific uses. In 1998, the FDA approved ACE for use in beverages. In the EU ACE is authorised for food use with exception for foods for young children aged 1–3 years. The daily intake of this sweetener is 9 mg Kg^{-1} body weight, confirmed by the European Union (EU) Scientific Committee on Food (SCF) in 2000 (EFSA, 2016). (Shankar et al., 2013)

ACE is 200 times sweeter than sugar and has no calories because it is not metabolised by the body and thereby excreted unchanged (Whitehouse et al., 2008). However it provides bitter taste at high concentrations, therefore it is usually blended in food products with other sweeteners, such as aspartame. It can be found in baked goods, frozen desserts, candies, beverages, cough drops, and breath mints (Pierini et al., 2013).

This compound has a very high stability. Samples kept at room temperature for more than 6 years and either exposed to light or protected from it did not show any signs of decomposition or differences in analytical data compared with freshly produced material. It is a very polar component even at room temperature, ACE dissolves readily in water. The solubility at 20°C is about 270 g/l water (Nabors, 2004)

1.3. Omics tools.

As it has been mentioned above, detection of PCPs at very low concentrations due to the advances in analytical techniques, has revealed a worldwide issue and thereby many studies have been done on their occurrence and presence (Díaz-Garduño et al., 2017). In fact, PCPs have been detected in traditionally not polluted areas or places far from the main source of contamination, such as the Polar Regions (Esteban et al., 2016; González-Alonso et al., 2017). However, very little is known about the ecotoxicological effects of PCP in aquatic or terrestrial organisms (Berset and Ochsenbein, 2012).

Since the environmental exposure of emerging contaminants is totally different to the last century discharged pollutants, a new mechanism is needed to provide targeted high-quality monitoring information on the possible negative effects of the most relevant EPs for environmental risk assessment (Kalogerakis et al., 2015). Nowadays contaminants are released in low concentration but during long time or chronically. Chronic low dose effects, however, may not cause overt toxicities but may cause adverse ecological outcomes in terms of population levels and biodiversity through rather subtle changes in the health and physiology (eg, behavior) of the organisms (Hampel et al., 2016a).

Only few studies have been conducted in order to analyse the potential toxicity of UV filters and artificial sweeteners in organisms. For instance Soh et al. (2011), showed that sucralose does not appear toxic to sugarcane plant growth at low concentrations. However the authors suggested that long term low dose exposure might lead to chronic risk with largely unknown consequences for environmental health. Similar recent studies have proved that physiology and locomotion of

Daphnia magna and other crustaceans such as gammarids and mysid shrimps were influenced by exposure to sucralose. The authors of these studies considered this divergence from normal behaviour as a matter of concern and suggested using screening technologies (e.g. high throughput omic techniques) to evaluate the potential of EPs to elicit effects in aquatic organisms at molecular levels which is the first response to stress. (Huggett and Stoddard, 2011; Tran et al., 2013; Wiklund et al., 2012)

In order to detect low concentration exposure effects, molecular and cellular tools have been used recently by the scientific community to evaluate and monitor the impact of emerging contaminants on marine wildlife. In particular high-throughput screening techniques or “omics” tools have begun to influence environmental science in a very significant way fifteen years ago (Denslow et al., 2012). Furthermore, the use of omic tools to evaluate the influence of the potential effects of pollutants in the environment will provide information about the Mode of Actions (MoA) of the substance that are functional or anatomical changes at cellular level of the organism after its exposure to a contaminant or substance (Hampel et al., 2016b). This will allow to extrapolate effects across species and reduce uncertainties in environmental risk assessment (Benson and Di Giulio, 2006).

The term “omics” indicates “a totality of some sort”. In life sciences it is used to measure some characteristic of a large family of cellular molecules, such as genes, proteins, or small metabolites and can be divided into three main categories: genomics/ transcriptomics, proteomics, and metabolomics/metabonomics (TABLE X). Omic profiling is the measurement of the activity or expression of thousands of genes (transcriptomics), proteins (proteomics), or metabolites (metabolomics/metabonomics) in order to understand the behavior of cells, tissues, organs, and the whole organism at the molecular level. These technologies have the potential to facilitate the development of a predictive toxicology study (Aardema and MacGregor, 2002; Hampel et al., 2016a, 2015).

The use of omic technology in toxicology (toxicogenomics) was initiated after the development of the first high-density techniques (microarrays). However, excitement surrounding this new technology generated “hype” that yielded unrealistic

expectations of the timeline for their incorporation into risk assessment. There is now a more realistic understanding of the potential contribution of omics to toxicology (Van Aggelen et al., 2010).

Transcriptomics measures de gene expression of a chosen organism. It is the most used omics technique in environmental toxicology and it has been used to evaluate the effects of pesticides, industrial by-products, pharmaceutical and nanoparticles (Hampel et al., 2016b; Martyniuk and Simmons, 2016a).

Technique	Target	Information about
Transcriptomics	All transcripts (mRNA) in sample	Which genes are turned on/off
Proteomics	All proteins in sample	Which genetic information is translated into proteins
Metabolomics	Metabolites of cell	Which proteins are functioning in the cell

Table 1.3: Summary of the Most Used Omic Techniques in Ecotoxicology, the Analyzed Targets, and Expected Information (Hampel et al., 2016b)

Protein expression has been applied in ecotoxicological studies for decades by the quantification of proteins using techniques such as western blotting, radio- and ELISAs and enzyme activities assays (Martyniuk and Simmons, 2016a). Nowadays proteomics analyses the expression of all proteins in a sample with Liquid Chromatography combined with Mass Spectrometry (LC-MS) technologies mainly used for protein separation and identification (Dowd, 2012). In the field of ecotoxicology proteomics became an essential tool because not all RNA is translated into proteins and post translational modifications can induce changes in the final functional proteins that would not be appreciable only by transcriptomic techniques (Suárez-Ulloa et al., 2013)

Metabolomics is the scientific study of the metabolome. The metabolome is the complete set of endogenous metabolites that are the intermediate and final chemical

products made inside a living organism during metabolism and that can be modified due stress conditions (Martyniuk and Simmons, 2016a). The metabolome presents some advantages over the genome/ transcriptome and proteome for use in ecotoxicology. Although there are definitely exceptions among taxonomic phyla and across kingdoms, metabolite chemical structures are well conserved across diverse animal species, and thus, the difficulties of non-model organisms are easier to resolve in toxico-metabolomics. Additionally, bio-fluids contain many metabolites and serve an ideal sample medium because extraction methods are simpler, non-lethal sample collection is a potential option, and thus repeated measures that can be performed more easily to study temporal effects (Martyniuk and Simmons, 2016b; Veldhoen et al., 2012). Within the metabolomic area it can also be measured the xenometabolomes, which are the metabolites of foreign substances such as drugs or pollutant (Crockford et al., 2008). The identification of these biotransformation products are in the scope of the present work.

Non-model organisms are organisms that have not been selected by the research community for extensive study either for historic reasons, or because they lack the features that make model organisms easy to investigate. Nevertheless nowadays they are considered as emerging systems for tackling questions across the whole spectrum of biology (Russell et al., 2017).

Taking all the aforementioned into consideration the **main objective** of the present work the identification of xenobiotics and their metabolites (the xenometabolites) (Al-Salhi et al., 2012) in a locally representative bivalve, the Manila clam *Ruditapes philippinarum* in order to understand the biotransformation process of two PCPs, acesulfame-K an artificial sweetener and 4-MBC a UV filter and the possible adverse effects in the exposed organism.

Additionally two different extraction protocols will be compared in order to provide the most suitable extraction methods for the analysis of xenobiotics and their metabolites.

The Manila clam was selected not only for its fundamental role in the marine ecosystem, but also for its commercial value in aquaculture industry. The production of this specie reached approximately 4 million tonnes in 2013 (Yamasaki et al., 2016).

Additionally, this group of organisms displays key features legitimizing their application as sentinel organisms for the biomonitoring of harmful compounds, particularly in coastal and estuarine areas, including: ubiquitous distribution, easy accessibility, filtering lifestyle, as well as strong resistance to a wide range of pollutants (Fig. 1.4.) (Suárez-Ulloa et al., 2013)

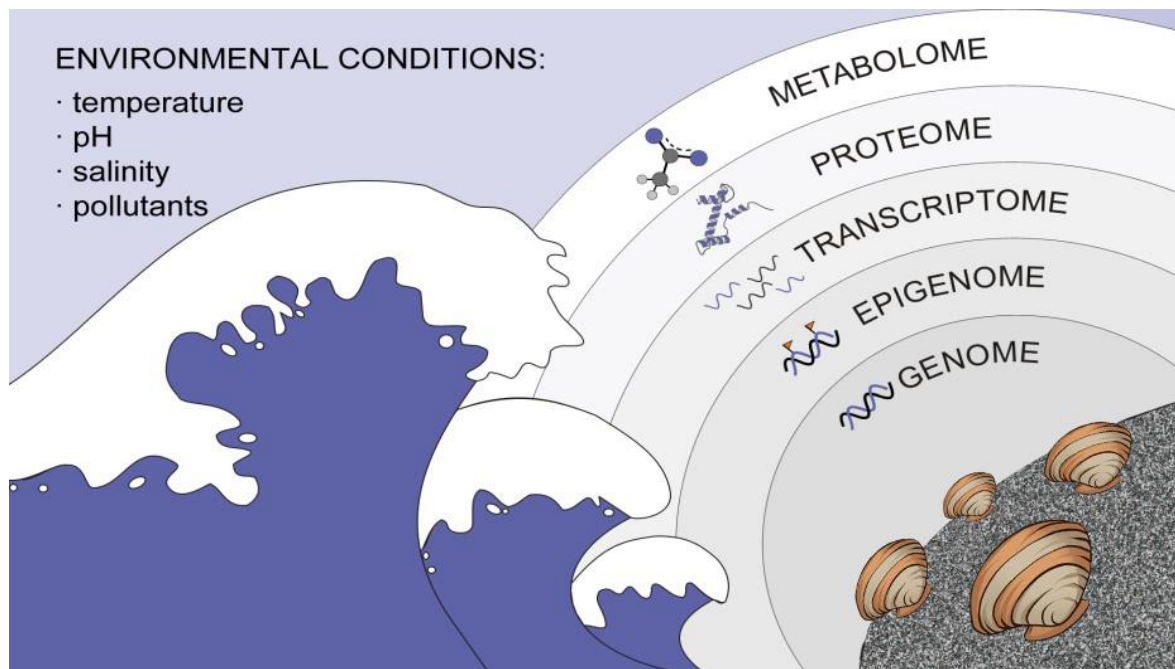


Fig. 1.4: Representation of how environmental conditions and harmful effects can have associated responses in different molecular levels of marine bivalves. Modified from Suárez-Ulloa et al., 2013

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Chapter 2

Materials and methods

2. Material and methods.

In the present work a laboratory assay was conducted with the manila clam *Ruditapes Philippiarum* exposed to an artificial sweetener, Acesulfame-K (ACE) and a UV filter, 4-methylbenzylidene camphor (4-MBC) followed by the appropriate dissection of tissues, extraction and analysis of metabolites that will be explained below.

2.1. Manila Clam Exposure

Adults individuals of *Ruditapes philippinarum* (Manila clams) were purchased from commercial clam fishery Cetarea del Sur (Cadiz, SW Spain) and transferred to the aquiculture facilities of the University of Cadiz (Faculty of Marine and Environmental Sciences) where the experiment took place during the month of July 2016 (University of Cádiz, UCA).

Before exposure, the clams were acclimated in aerated saltwater tanks for 1 week in a temperature controlled room at 20° C fed with isochrysis galbana ad libitum every day. The exposure experiments were conducted in 50L fiberglass tanks (60 x 60 x 13 cm) filled with filtered seawater and 50 individuals per tanks along 10 days: 7 days of exposure (uptake period) and 3 days of depuration. Clams were exposed to nominal concentrations of 1, 10 and 100 $\mu\text{g L}^{-1}$ of 4-MBC and ACE-K and appropriate control (0 $\mu\text{g/l}$) for both treatments and solvent control (only ethanol) for 4-MBC treatment due to the low solubility of this compound in water. Every treatment was run in triplicates. An additional tank with no organisms was used to monitor daily 4-MBC and ACE-K concentration in water.

The ACE-K experiment was run under flow-through conditions (in/out flow: 250 mL min^{-1}) figure 2.1. On the other hand, the 4-MBC experiment was conducted under semi-static conditions due to the high hydrophobicity of the compound that hampered the functioning of peristaltic pumps. Thus, water was changed daily in the 4-MBC experiment and a constant volume of 5 μL of 4MBC stock solution, was added to each tank for 7 days whereas ACE-K was added by a pumping system at a flow rate of 1 mL min^{-1}



Fig. 2.1: **A)** Semi-static exposure experiment with 4-MBC, and **B)** flow-through system experiment with ACE-K with Manila clams. Modified from Tonini (2017)



Fig. 2.2: Clams samples during the lyophilisation process (Tonini 2017).

2.2. Metabolite extraction

The extracted samples were used to identify the possible metabolites (the xenometabolome) of the selected contaminants metabolized by the manila clam. Metabolites were extracted using the procedure described by Weckwerth et al. 2004 (fig. 2.3), a protocol for the simultaneous extraction of metabolites, proteins and RNA. This

Tanks were regularly checked to remove debris and dead clams that could interfere with the experiment. Samples were collected at day 0, 1, 3, 5, 7 and 10. During the sampling process all samples were snap frozen in liquid nitrogen and stored at -80°C until further analysis apart from the samples containing the whole clam tissue which were kept at -20°C in glass bottles, and finally lyophilised for about 60 hours to remove water content (Fig. 2.2). Clam tissues were milled and homogenized with a mortar prior to extraction and analysis.



Fig. 2.4: process of disassociation of metabolites from membrane and cell wall components

protocol has been implemented in its first steps with the aim of following up this thesis with future research work on gene and protein expression in the same sample. Modifications in the protocol were performed according to the objectives. (Salem et al., 2016; Simões et al., 2013; Valledor et al., 2014; Vorreiter et al., 2016)

A volume of 4 mL of a single phase solvent mixture of methanol/chloroform/water 2.5:1:1 v/v/v kept at -20°C was added to the yophilised clam tissue, homogenised with an Ultra-Turrax for 45 seconds. and thoroughly mixed at 4°C for 30 min to disassociate metabolites from membrane and cell wall components (fig. 2.4) (Weckwerth et al., 2004). After centrifugation during 20 minutes at 14,000 G and 4°C, the supernatant was transferred to a new tube and kept at 4°C. The remaining pellet was extracted in a second step with 4 mL of a single phase solvent mixture 2 methanol/chloroform 1:1 v/v at -20°C, to make sure that the maximum amount of metabolites (both polar and nonpolar) were extracted. The same procedure as explained above was followed with the second organic solvent extract. In order to separate hydrophilic (polar) metabolites from lipophilic (nonpolar) metabolites for their proper analysis, the chloroform phase was separated from the methanol phase by adding 1ml of water (molecular grade). The two phases were clearly defined by a sharp interphase (fig. 2.5). Polar and nonpolar metabolites, presumably upper and lower layers respectively were transferred to new tubes (fig. 2.3).

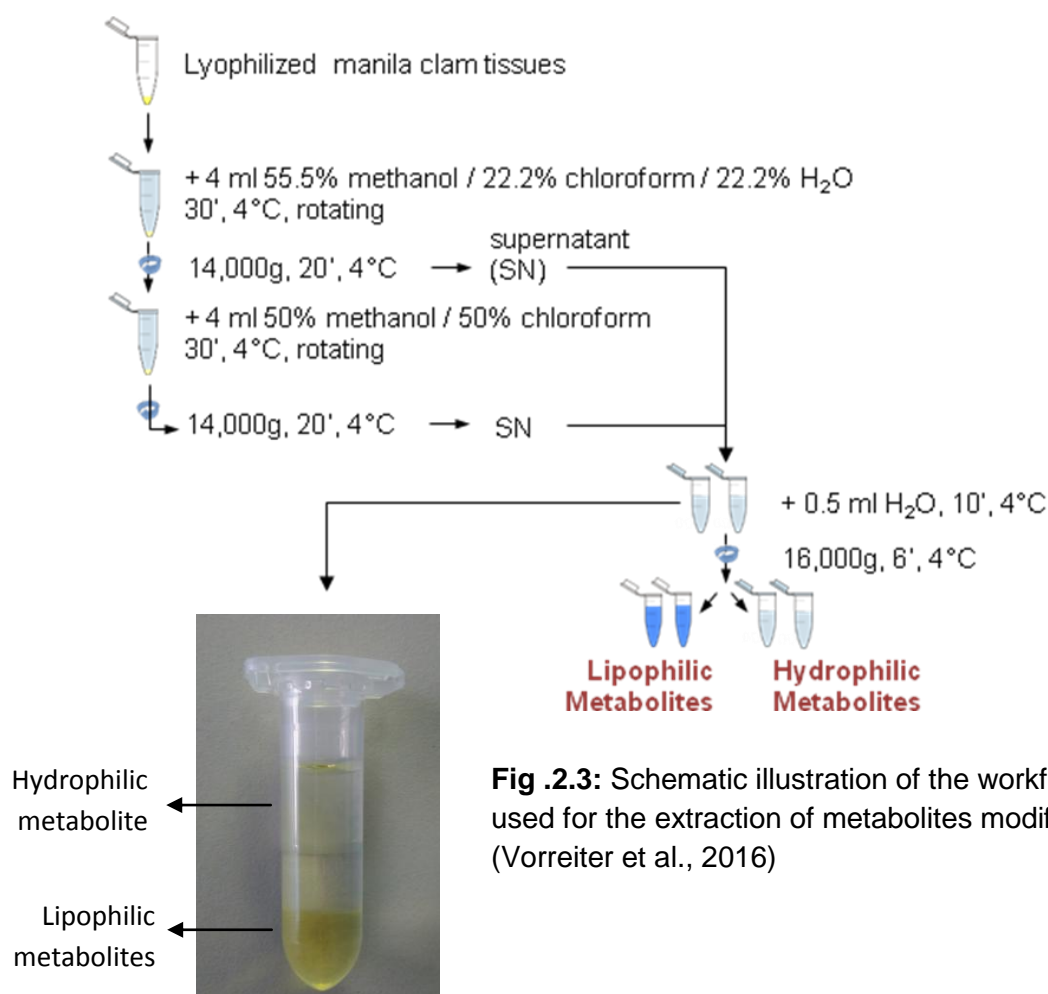


Fig .2.3: Schematic illustration of the workflow method used for the extraction of metabolites modified from (Vorreiter et al., 2016)

According to previous studies performed by the research group (Pintado-Herrera et al., 2014; Tonini, 2016), and previous tests done with our samples, metabolómica analysis of Acesulfame-K was conducted by Liquid Chromatography (LC) whereas Gas Chromatography was used for 4-MBC instead. Some authors (Harris, 2014) suggest that, according to their definition, metabolites are quite similar to the parent structure, and thereby they share properties. For this reason, the identification of ACE- K metabolites was performed only in the polar phase in contrast to the identification of 4-MBC metabolites.

Before injection either in the LC or GC system solid-phase extraction was implemented to our samples to minimize



Fig. 2.5: sharp interphase of both organic solvent extract

interferences. The general SPE procedure aims to provide sample extracts that are free of interfering matrix components and concentrated enough for detection. The SPE process basically consists in four different steps: conditioning, sample addition, washing and elution (Lucci et al., 2012) (Fig. 2.6)

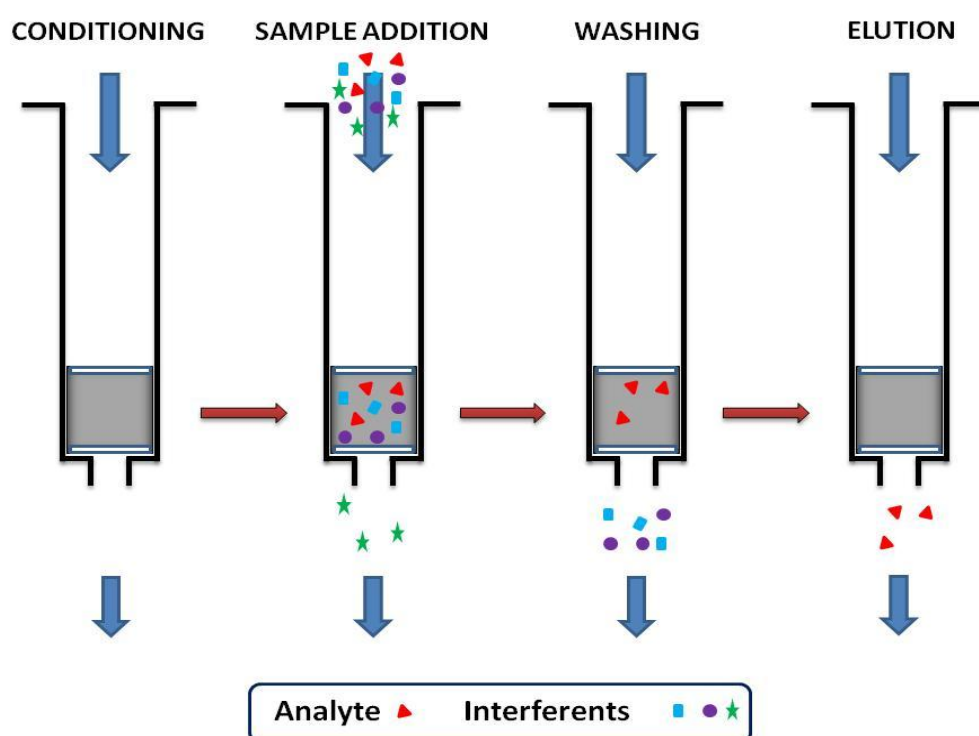


Fig. 2.6: Schematic representation of SPE clean-up procedure.(Lucci et al., 2012)

First, the most suitable solid sorbent is selected and conditioned using an appropriate solvent. The choice of sorbent is a key factor because this can control parameters of primary importance such as selectivity, affinity and capacity (Nováková and Vičková, 2009). During the conditioning the functional groups of the sorbent bed are solvated in order to make them able to interact with the sample. Additionally, the condition phase has also the purpose of removing potential impurities that can contaminate the sample and eliminates the air present in the column filling the void volume with solvent. The sample addition consists in the percolation of the samples through the solid sorbent (cartridge). During this step, the analytes as well as some matrix components are retained and thus concentrated on the SPE packing material. Successively, the analytes and interferences separation could be realized by the three following ways: selective extraction, selective washing

or selective elution. Selective extraction is performed when the SPE procedure is used to remove the interfering components (trace enrichment). In this way, only selected components are retained. Selective washing is realised when the target analytes and the impurities are retained on the sorbent bed: the impurities will be eliminated with wash solutions that are strong enough to remove them, but weak enough to leave the analytes behind. Selective elution consists in the elution of the adsorbed compounds of interest by a solvent that leaves the strongly retained impurities behind (Lucci et al., 2012). Since the compound that we needed to analyse had very distinct properties, different method for separating analytes and interferents were used.

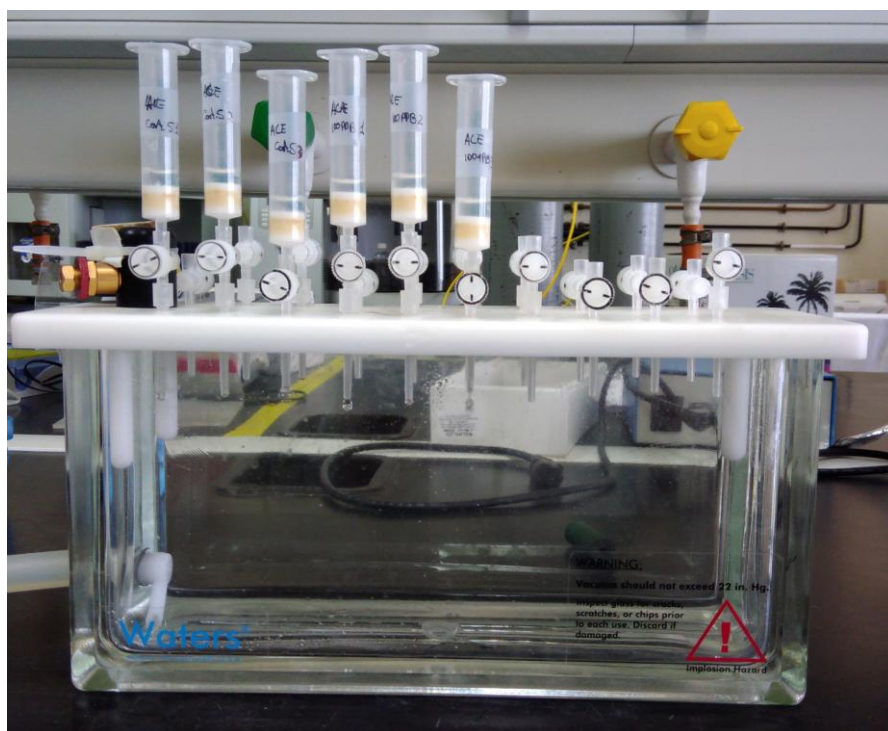


Fig. 2.7: Manual Solid Phase Extraction aspirated by vacuum.

For the samples containing the artificial sweetener **Acesulfame-K** Oasis® HLB (Hydrophilic Lipophilic Balanced) mini-columns extraction cartridge were used. These columns allow keeping the polar compounds and discarding the nonpolar (Kettle, 2013). The extracted sample was first mixed with 10mL of acidified MilliQ water at pH = 2. Then the HLB column was conditioned with 5 mL of methanol followed by 5 mL of MilliQ water at pH = 2. After conditioning, the sample was added to the cartridge with 3 mL of MilliQ water at pH = 2. Then, the column was dried for

10 min, and eluted with 5 mL methanol were the analytes were in suspension (fig. 2.7) (Álvarez-Muñoz et al., 2014; Baena-Nogueras et al., 2016).

For the samples containing the UV filter **4-MBC**, Supelclean™ LC-Si SPE Tubes were used, filled with silica. These columns are appropriate for hydrophobic compounds because, unlike the previous ones, they eliminate polar compounds and stay with non-polar ones, such as the lipids. Additionally silica has the highest capacity for lipid retention compared to alumina, Florisil or C18 adsorbents (Kettle, 2013). In this case the column was conditioned with a mixture of hexane/dichloromethane 2:1 v/v. Then, the sample was added to the column and cleaned with 40 mL of the mixture hexane/dichloromethane collecting the resulting extract (fig. 2.8)



Fig. 2.8: Manual Solid Phase Extraction aspirated by vacuum with Silica columns while collecting the extract

Then, in both cases, the resulting extracts were evaporated to dryness using the XcelVap Evaporation/Concentration System (fig. 2.9) adjusting the variable pressure profile from 12 to 24 psi and a temperature of no more than 45 °C. The dried samples of ACE were resuspended on 500 µL of methanol/Water (25:75) and the samples of 4-MBC in 500 µL of ethyl acetate.

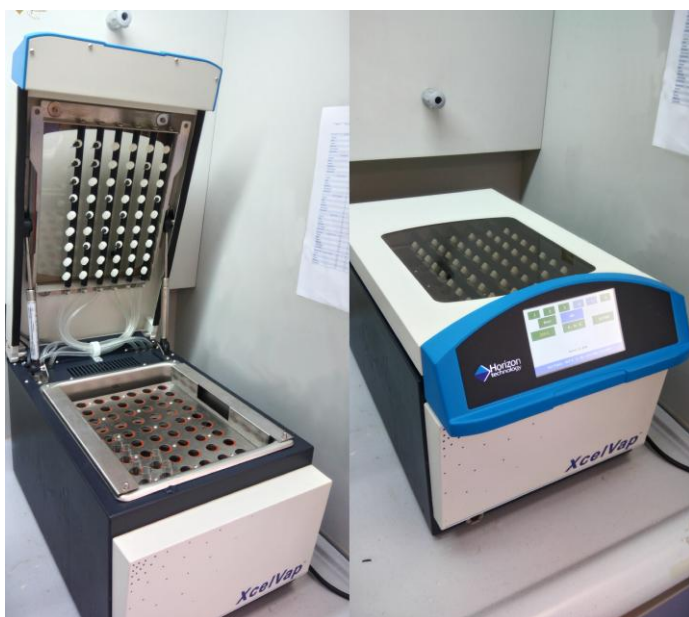


Fig. 2.9: evaporation of the samples for its pre-concentration.

Finally all samples were sonicated in a sonicating bath for 10 mins and filtered in Eppendorf vials containing PTFE centrifuge filters (0.22 μm pore size), and centrifuged at 7000 g for 10 minutes to eliminate suspended particles.

2.3. ACE metabolites Identification.

- Primary ion formation

$$e^- + N_2 \longrightarrow N_2^+ + 2e^-$$
- Secondary ion formation

$$N_2^+ + H_2O \longrightarrow N_2 + H_2O^+$$

$$H_2O^+ + H_2O \longrightarrow H_3O^+ + HO$$
- Proton transfer

$$H_3O^+ + M \longrightarrow (M+H)^+ + H_2O$$

fig. 2.10: Ionization process of ESI technique.

Analysis of the compound was carried out by ultra-performance liquid chromatography time-of-flight mass spectrometry (UPLC-QTOF-MS) using a Waters Xevo® G2-XS QToF with an ElectroSpray Ionization (ESI) (fig. 2.10) source operated in either positive or negative mode. Sample extracts (5 μL) were separated by an Acquity UPLC system (Waters, UK) using an ACQUITY UPLC® HSS T3 column (2.1x 100 mm, 1.8 μm

particle size, 40°C). Mobile phase solvents were 95% water + 0.1% formic acid (A) and 5% methanol (B), in a initial ratio (A:B) of 95:5. The flow rate was 0.4 mL min⁻¹ with the following linear gradient: 0.2- 0.7 min from 5-90% B, from 0.7-4.20 min 90-100% B, from 4.20-6 min 100%B and 6-7.20 min 100-5% B.

The ionisation parameters were: capillary voltage (\pm) 0.7 Kv, cone voltage 30 V, source temperature 120°C, desolvation temperature 450°C and cone gas and

desolvation nitrogen flow 850 L/h. The collision energy was 15 eV. Leucine enkephalin (parent $m/z= 554.2615$ and fragment $m/z= 236.1035$) was used as the lock mass standard allowing constantly mass correction in order to achieve mass accuracy of analytes even at the edge of the detection limit (Liu et al., 2007; Moco et al., 2006).

Mass spectra were collected in full scan mode at 100-800 m/z . Spectral peaks were analysed using Masslynx software (version 4.1 SCN901, Waters Corporation, MA, USA). For the identification of metabolites, Metabolynx XS (post-acquisition data processing software) was used (Waters Corporation, MA, USA). Open source databases were used to search the structural identities of molecules (Human Metabolome database, KEGG LIGAND, LIPIDS MAPS, Pubchem, Chempider, Golm Metabolome Database (GMD) and METLIN).

2.4. 4-MBC metabolites Identification

The separation and identification of 4-MBC metabolites was carried out by gas chromatography tandem mass spectrometry (GC-QTOF-MS), using gas chromatography (SYNAPT-G2-Waters) with Atmospheric pressure chemical ionization (APCI) process (fig. 2.11).

Capillary gas chromatography analysis was carried out on a BR-5MS column (30 m x 0.25 mm i.d. x 0.25 μm film thickness), keeping the carrier gas flow (helium) at 2 mL min^{-1} , and the transfer line and the injection port temperatures at 280° C. The column temperature ramp was as follows: 150° C for 2.5 min, increased at 25° C min^{-1} to 190° C, then at 10° C min^{-1} to 320° C, and held for 3 min. Injection volume was 1 μL in split mode.

Mass spectra were collected in full scan mode at 100-800 m/z . Spectral peaks were analysed using Masslynx software. For the identification of metabolites, Metabolynx XS was used. And the same databases were used to search the structural identities of molecules.

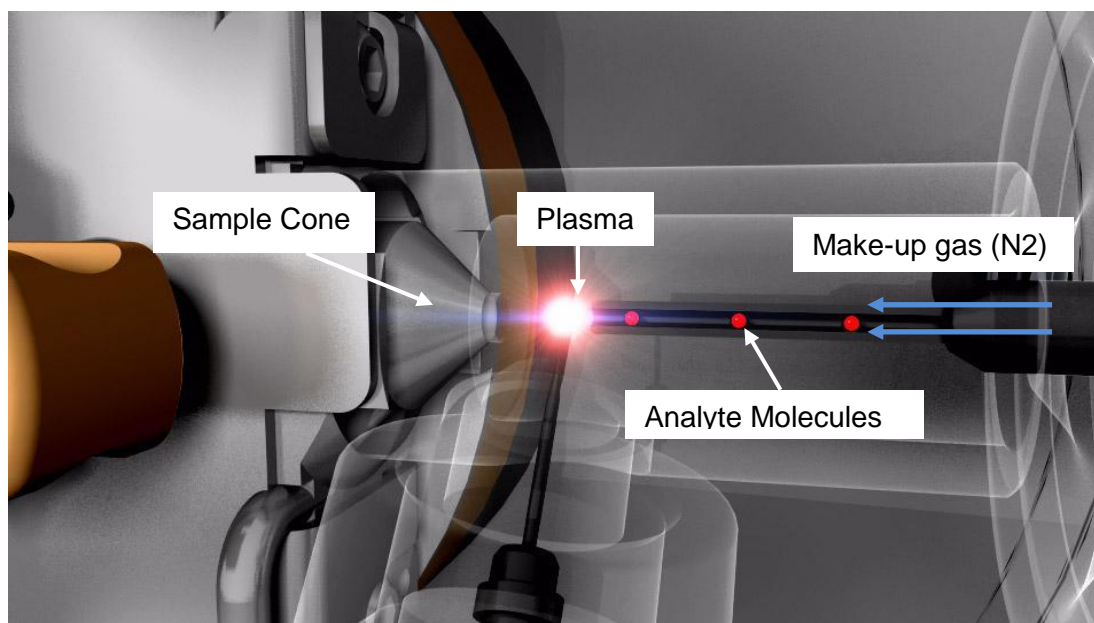


Fig. 2.11: APCI ionisation Source. (Modified from Waters, 2013)

2.5. Metabolyx™ processing approach

Post-acquisition analyses were performed using a MetaboLynx™ (v4.1) program (Waters Corp., Milford, MA, USA) under the operating interface of Masslynx by automatically comparing MS data from the dosed analytes sample and the blank control sample. An extensive list of potential phase I and phase II biotransformation reactions (e.g. hydroxylation, methylation), in combination with the elemental compositions of the substrate molecules were selected for the predicted metabolites scanning, to generate a series of extracted ion chromatograms (EICs). These EICs are compared between the control and sample to eliminate those chromatographic peaks in the sample that also appear in the control with a significant abundance (Zhang et al. 2017; Guo et al. 2010; Guo et al. 2011)

MetaboLynx™ processing settings for each possible biotransformation (table 1 in annexes) were generated within a 0.05mTh window of the mass of the test compound [M+H] ion in GC and [M-H] in LC and were smoothed once with a window size of 2 scans. Peaks with an area of less than 5 peak area unit (p.a.u.) were eliminated. Control and analyte samples were processed in the same way, and the

integrated peak lists from the EICs of analyte samples were compared with those from the corresponding control samples.

The other method parameter settings were as follows: analysis retention time range was 0-24 min for GC and 0-7.5min for UPLC; retention time tolerance was 0.1min; mass range was settled as full acquisition; mass tolerance was 0.5 mDa; noise elimination level was 5; peak relative intensity threshold was 14; the spectrum was above the relative intensity of 3 %.

In the same retention time, peaks present in the exposed sample while absent in the control sample or peaks that absolute area in the drug-containing sample was 5 times greater than that in the control sample were extracted as metabolite candidates.

2.6. Draw of molecular structures of identified metabolites

The proposed chemical structures of the found metabolites were created using ChemDraw® Professional, Version 16.0, PerkinElmer Informatics <http://www.cambridgesoft.com/software/overview.aspx>.

In order to propose a possible position for the identified biotransformations, a computational model that predicted the specific position of metabolism and reactivity transformations in small molecules was used (Hughes and Swamidass, 2017) <http://swami.wustl.edu/xenosite/>.

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CHAPTER 3

Results

3. Results

The post-acquisition data were processed using MetaboLynx. With key parameters carefully set, MetaboLynx is able to show the presence of a wide range of metabolites from complex data variables with only limited requirement for manual intervention and data interpretation time (Guo et al. 2011; Guo et al. 2010).

Data evaluation with MetaboLynx performed in this study can be considered to be a three-stage process. Firstly, the acquired data were processed using a user-defined parameter file, to generate a preliminary report file. This involves an automated comparison of analytes GC-LC/MS chromatograms with appropriate control samples, with user-defined settings for integration of extracted ion chromatograms (EICs) and reconstruction of spectral data. Secondly, this report is displayed in the browser, and the output is refined by a variety of data filters. Finally, the large numbers of peaks generated were inspected manually to determine whether they are likely to be compound-related metabolites or not. In order to accept the EICs as a metabolite, the peak area in the analyte had to be at least 5 times greater than that of the control (Guo et al. 2010)

3.1. Separation and identification of metabolites of 4-MBC

First of all, the levels of the pollutant was checked in water samples of the control aquaria ($0.01\text{--}0.05\ \mu\text{g L}^{-1}$) and in the control clam tissues ($1010\ \text{ng g}^{-1}$) indicating the absence of contamination (Tonini, 2017)

The GC/MS chromatograms obtained from Manila clam tissues revealed a complex pattern of peaks showing the parent compound (4-MBC) at the minute 8.45 and 8.75 (fig. 3.1). These two peaks correspond to the cis/trans isomers of 4-MBC respectively (Plagellat et al. 2006). Six different metabolites of $\text{C}_{18}\text{H}_{22}\text{O}$ were identified from their molecular composition, accurate mass and retention time in Manila Clam tissues. All the identified metabolites were present in the three triplicates. Table 3.1 summarises the main characteristics of the identified metabolites.

Table 3.1: Characteristics of metabolites of 4-MBC identified in Manila clam tissue after 7 Days exposure experiment and the parent compound in bold.

Observed value (m/z) $\pm 0.1\text{mDa}$	Retention Time (min)	Experimental molecular formula of ion	Theoretical mass	Δ (ppm)	Chemical identity	Mass difference	Intensity of metabolite signal
255.1749	8.45/8.75	C₁₈H₂₂O	254.1671	-0.5	4-MBC/ Parent compound	0.0003	8.26e⁶
257.1906	8.06	C ₁₈ H ₂₄ O	256.1827	0.3	Reduction	2.0157	8.01e ⁵
271.1698	8.64/9.52/10.01	C ₁₈ H ₂₂ O ₂	270.1620	-1.4	Hydroxylation	15.9950	1.94e ⁶
287.1649	9.83	C ₁₈ H ₂₂ O ₃	286.1569	-0.7	2x Hydroxylation	31.9896	1.42e ⁵
269.1536	9/9.93/10.25/10.42	C ₁₈ H ₂₀ O ₂	268.1463	0.3	Hydroxylation + Desaturation	13.9793	3.21e ⁵
285.1479	10.38	C ₁₈ H ₂₀ O ₃	284.1412	0.5	Quinone formation	29.9743	2.06e ⁴
299.1645	11.30	C ₁₉ H ₂₂ O ₃	298.1569	-1.0	Quinone formation + Methylation	43.9895	1.69e ⁴

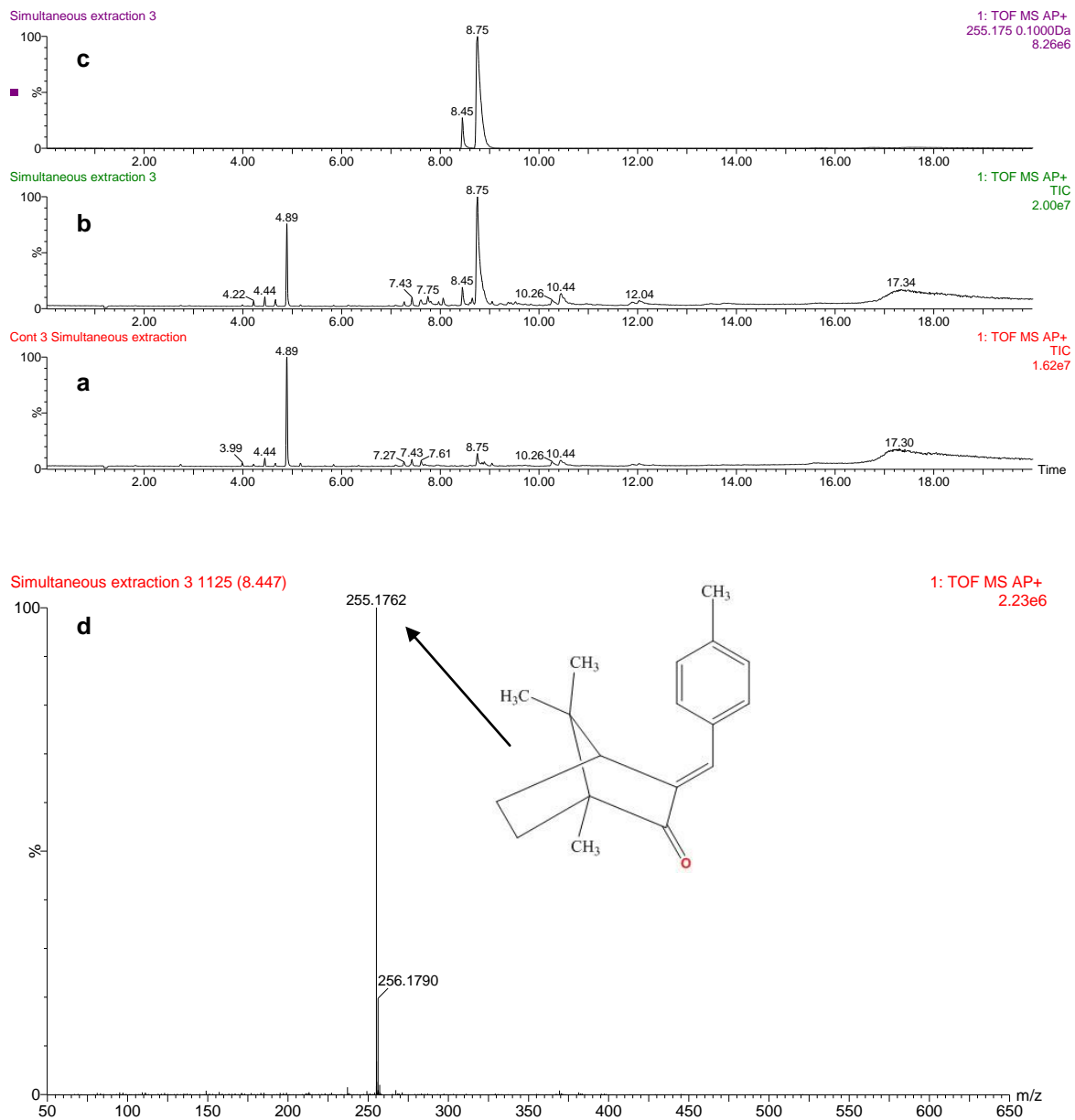


Fig. 3.1: Chromatograms of the control sample (a), the exposed sample (b) and the parent compound (c) (4-MBC) followed by the spectrum of the parent compound (d) (C₁₈H₂₂O) and its molecular structure.

3.1.2 Classes of metabolites identified and their comparison with two different extraction methods.

In order to determine the suitability or selectivity of the proposed extraction method disassociating the metabolites from the rest of the other cell compounds, a comparison between two different extraction methods has been performed. The same number of Manila clam samples from the same laboratory experiment were processed and analysed with Metabolynx™ in the same way as previously explained. The only difference between the two sample sets was the applied extraction protocol. This comparison was only performed in the samples exposed to 4-MBC, since just one metabolite were unambiguously identified in ACE exposed samples. The extraction protocol proposed in this work is a **simultaneous extraction** method, where metabolites, proteins and ARN can be extracted from the same sample (Weckwerth et al. 2004; Valledor et al. 2014; Vorreiter et al. 2016) while the other method is a very well established protocol optimized by the research group (Pintado-Herrera et al. 2016; Pintado-Herrera et al. 2013). This well established method is selective pressurized liquid extraction (**SPLE**) or in-cell clean-up PLE, very well known for its efficiency, speed and automation capacity for different groups of organic pollutants in different matrixes (Ghosh et al. 2011).

In the SPLE processed samples no new metabolites were identify with significant abundance (area of the analyte peak five times higher than in the control samples) so the already identified metabolites in the simultaneous extraction samples were searched in the SPLE samples for its comparison. Table 3.2 shows the intensity of each identified metabolite in the two different extraction methods and the presence/absence of interferences.

Parent compound

First of all, the parent compound (4-MBC) $C_{18}H_{22}O$ with m/z 255.1749 was identified in the exposed samples extracted with both methods. The abundance was very similar in both cases or slightly higher in SPLE extraction (fig.3.2)

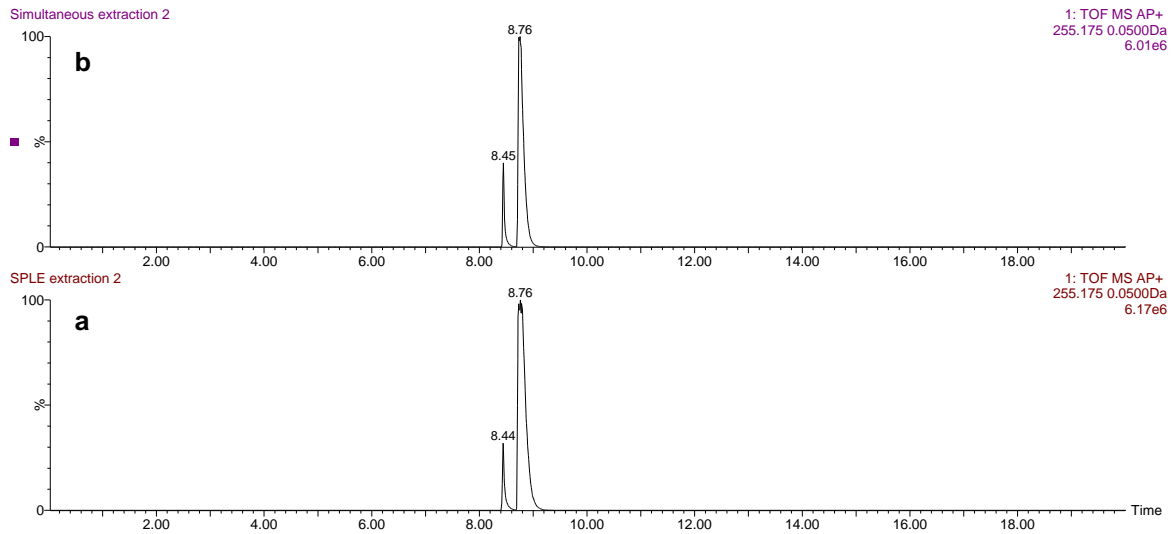


Fig. 3.2: Parent compound (4-MBC) in exposed samples extracted with both extraction methods: (a) SPLE method, (b) simultaneous extraction method.

Table 3.2: Comparison of the intensities of identified metabolites in both extraction methods and the interference identified

Metabolite name	Intensity in simultaneous extraction	Interferences in simultaneous extraction	Intensity in SPLE	Interferences in SPLE
Reduction	$8.01e^5$	no	$9.35e^5$	Palmitic acid ($C_{16}H_{33}O_2$)
Hydroxylation	$1.94e^6$	no	$2.07e^5$	Methyl palmitate ($C_{17}H_{34}O_2$)
2x Hydroxylation	$1.42e^5$	no	$4.41e^3$	Stearic acid or ethyl palmitate ($C_{18}H_{36}O_2$)
Hydroxylation + desaturation	$3.21e^5$	no	$6.74e^3$	Glycidyl palmitate ($C_{19}H_{36}O_3$)
Quinone formation	$2.06e^4$	Identified as $C_{19}H_{34}O_3$	Not present	Ethyl palmitate ($C_{18}H_{36}O_2$) and $C_{19}H_{34}O_3$
Quinone formation + methylation	$1.69e^4$	no	Not present	$C_{20}H_{31}O_2$, identified as a fatty acid

Reduction metabolite

In the analysed samples of the Manila clam exposed to 4-MBC, an intense peak was observed with m/z 257.1905 at RT 8.06 min. Based on its ionic composition and retention time it was unambiguously identified as a reduction of 4-MBC ($C_{18}H_{24}O$). It presents a high abundance in terms of detected metabolites. Fig 3.3 shows its chromatogram and spectrum.

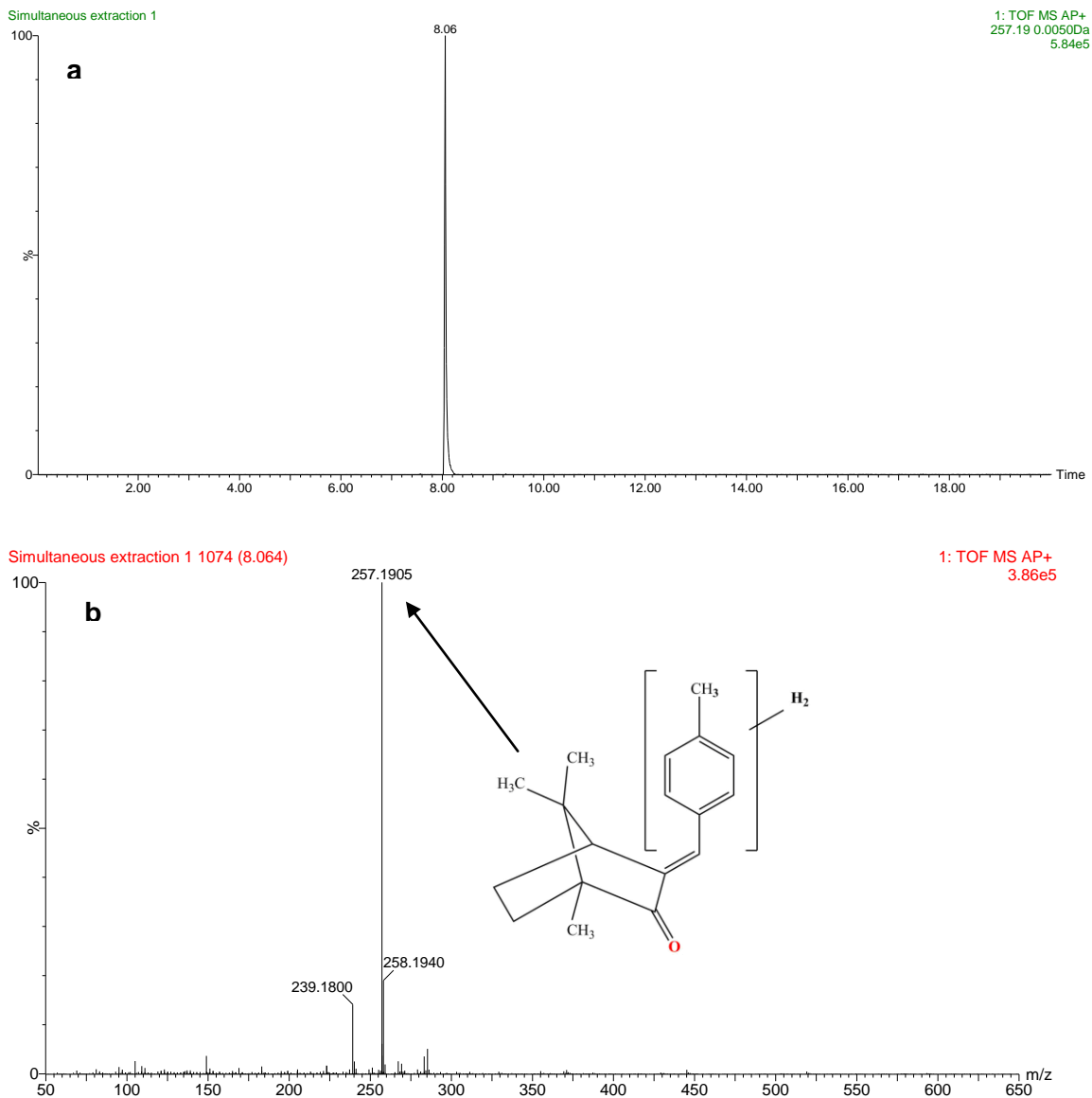


Fig.3.3: Chromatogram (a) and spectrum (b) of reduction metabolite with the proposed molecule structure in the simultaneous extraction method.

This metabolite appears with similar abundance or slightly higher in the SPLE (table 3.2). Interferences of palmitic acid ($C_{16}H_{33}O_2$) were present in SPLE extracted samples (fig.6.1 of the annexe). This fatty acid has been already identified in marine samples and suggested to come from marine organism (Tervahattu et al. 2002)

Hydroxylation metabolite

The second identified metabolite was the peak corresponding to m/z 271.1698, presence in the retentions times of 8.60, 8.64, and 9.52 and 10.01 min. This metabolite was identified as the hydroxylation of the parent compound by the conversion of a CH group into a COH group ($C_{18}H_{22}O_2$). Fig. 3.4 shows its chromatogram and spectrum with the proposed molecular structure. Their different retention times indicated different positioning of the hydroxylation group on the parent molecule changing the polarity and the elution behaviour of these analytes (fig. 6.2 of annexe) (Fasinu et al. 2014; Shen et al. 2010).

This metabolite present higher abundance in the simultaneous extraction than in the SPLE extraction samples (Table 3.2). Nevertheless in SPLE samples, the molecule m/z 271.2633 has such a high abundance that could “hide” the target compound due to the close m/z value (fig. 6.3 of annexe). This molecule has been identified as a derivative from palmitic acid ($C_{17}H_{34}O_2$ methyl palmitate) that has been identified in seawater since 1980 (Parrish 1988).

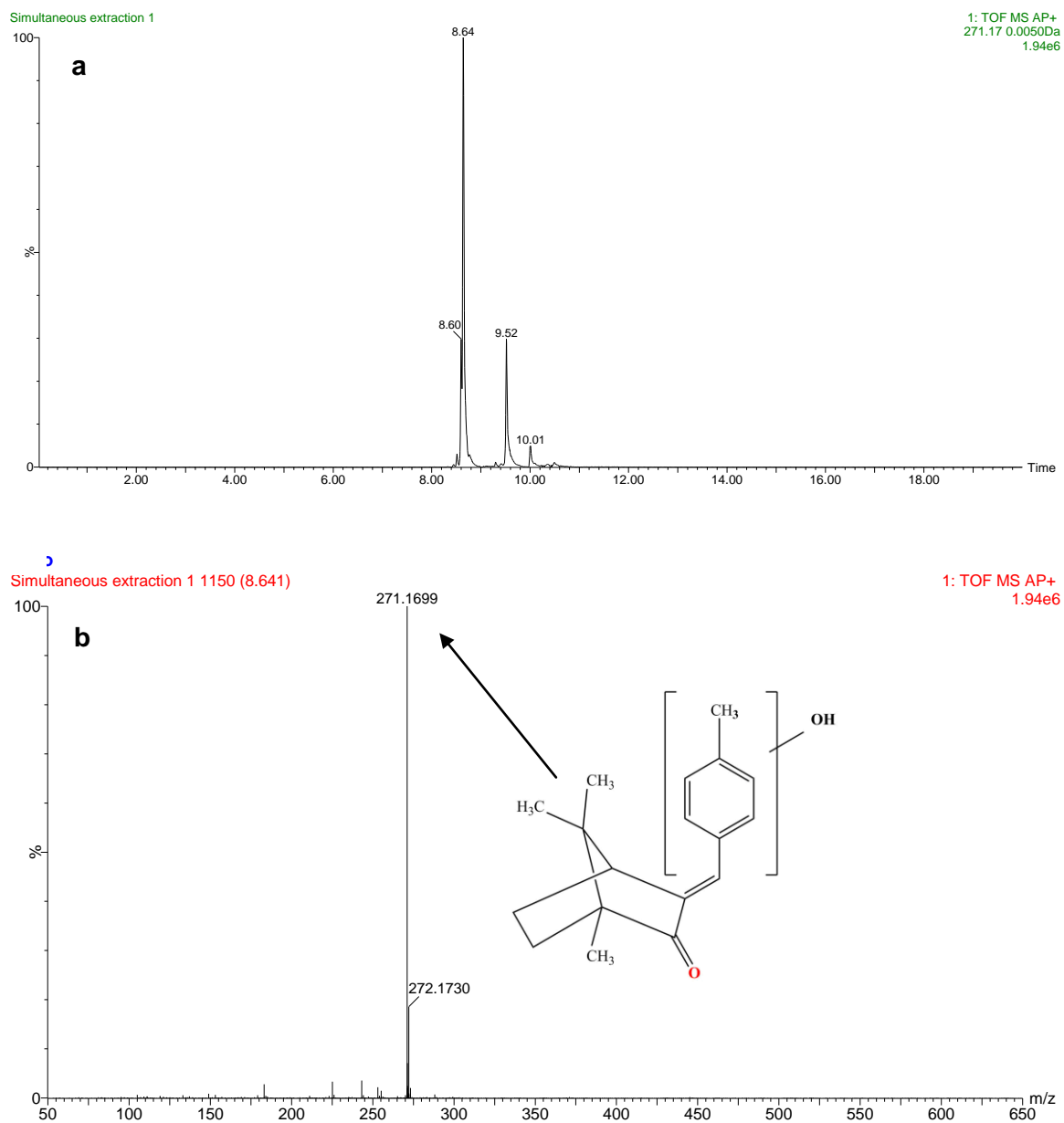


Fig. 3.4: Chromatogram (a) and spectrum (b) of hydroxylation metabolite with the proposed molecule structure in the simultaneous extraction method.

Double hydroxylation metabolite

Similar to the previous metabolite, the double hydroxylation biotransformation was identified with m/z 287.1649 at different retention times again due to the different possible positions of the hydroxylation groups (Zhu et al. 2005; Guo et al. 2011; Guo et al. 2010) (fig. 6.4 of annexe). Figure 5 shows the chromatogram and spectrum of this metabolite at different retention times with the corresponding molecule.

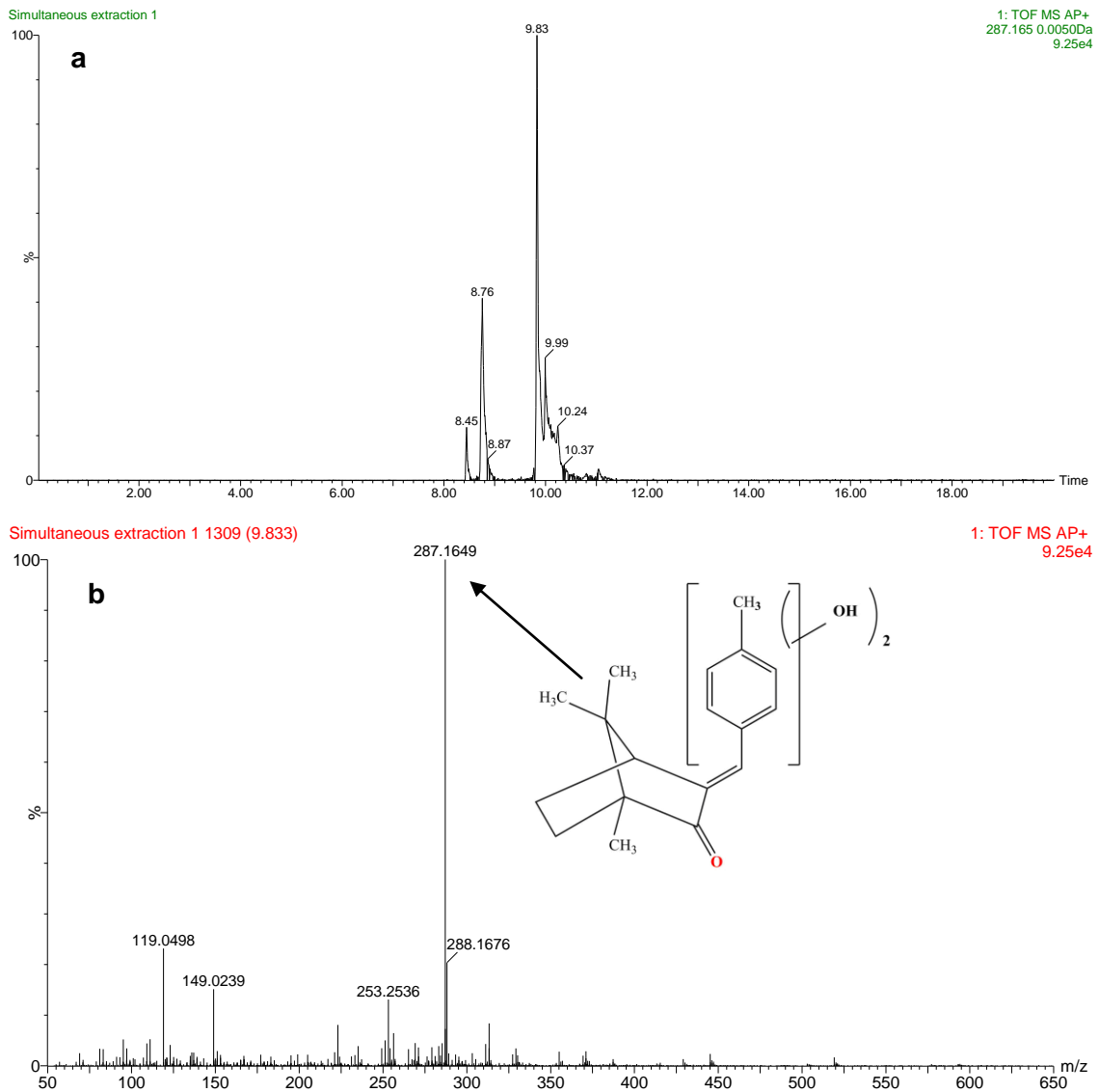


Fig . 3.5: Chromatogram (a) and spectrum (b) of double hydroxylation metabolite with the proposed molecule structure in the simultaneous extraction method.

This metabolite is identified at two different RT (8.76, 9.84 min) and although the first peak has higher intensity in SPLE samples, the general abundance of this metabolite seems to be higher again in simultaneous extraction samples than in SPLE extraction samples (Table 3.2.). At RT 9.83 min of the SPLE extraction the molecule m/z 285.2842 has been identified as interference (fig 6.5 of annexe). It was identified as $C_{18}H_{36}O_2$, proposed to be ethyl palmitate or Stearic acid.

Hydroxylation + desaturation metabolite

This metabolite was identified with m/z 269.1533. Fig. 3.6 shows its chromatogram and spectrum with the proposed molecule structure. Again it can be found at different retention times due to the different positions of the attached group. This metabolite was identified as a desaturation of the previously hydroxylated one, by removing the H_2 to form a double bond. At RT 10.42 min interference with high abundance of the molecule m/z 313.2736 was found (fig. 6.6 of annexe). This molecule seems to be glycidyl palmitate ($C_{19}H_{36}O_3$) (fig (Cheng et al. 2017)).

This biotransformation ($C_{18}H_{20}O_2$) was only found in SPLE samples at RT 10.42 min with lower abundance than in simultaneous extraction samples (fig.3.7). High level of interferences were detected in SPLE samples specially the molecule m/z 313.25 identified as $C_{19}H_{36}O_3$ that seems to be glycidyl palmitate (Cheng et al. 2017).

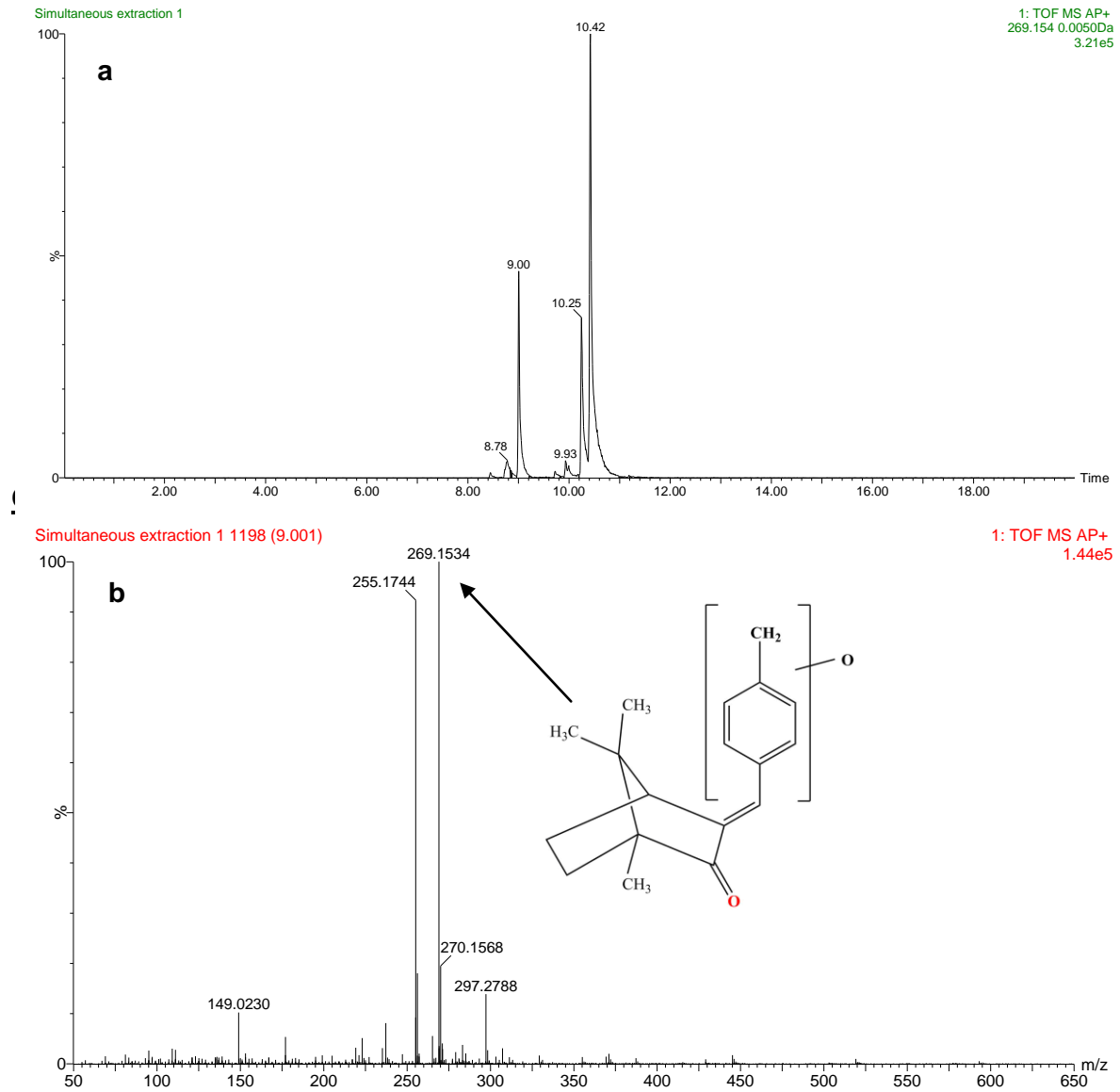


Fig.3.6: Chromatogram (a) and spectrum (b) of double hydroxylation + desaturation metabolite with the proposed molecule structure in the simultaneous extraction method.

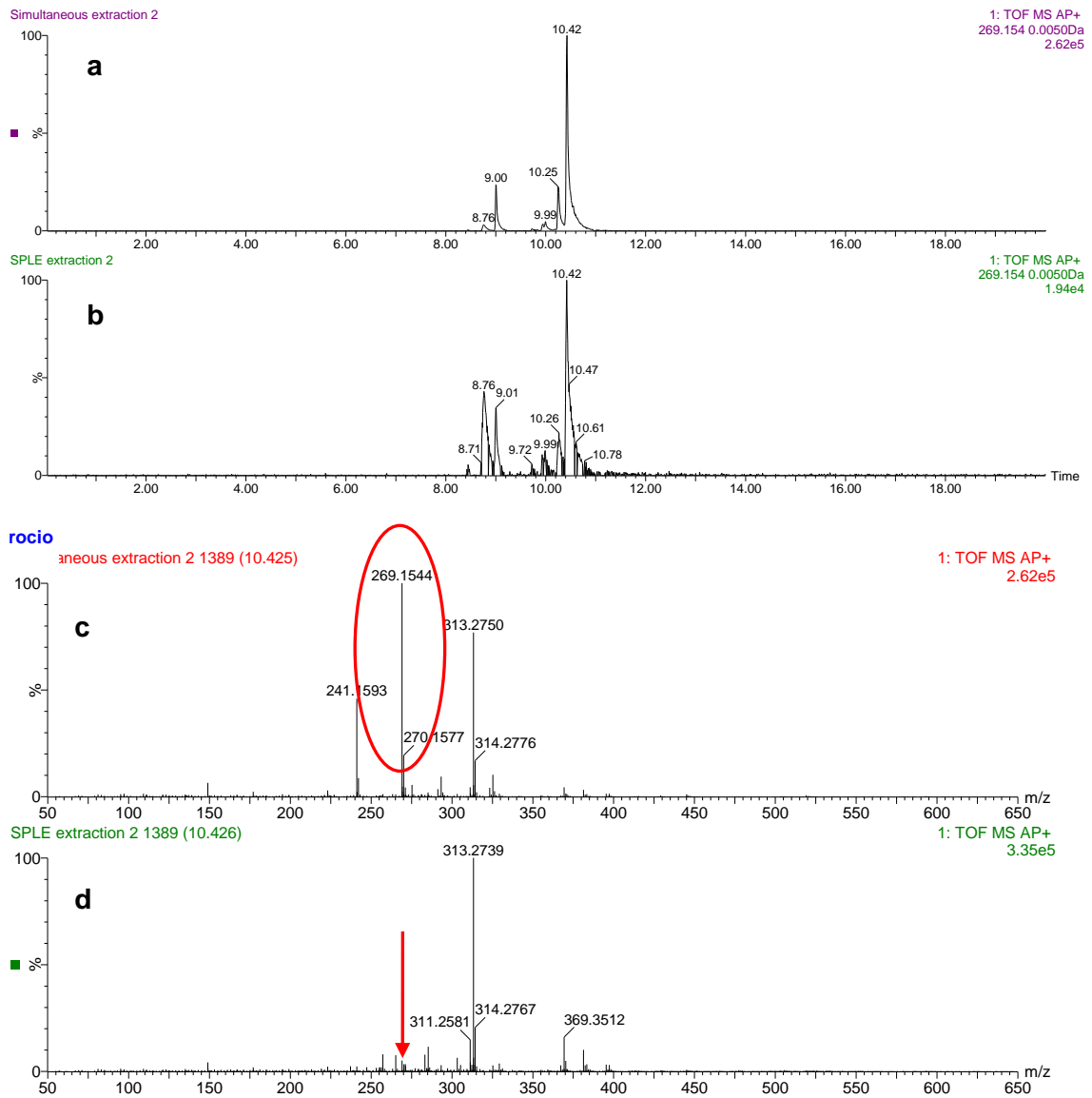


Fig. 3.7. Spectrum of simultaneous extraction (a) and SPLE (b) and chromatogram of simultaneous extraction (c) and SPLE (d) of hydroxylation + desaturation metabolite of 4-MBC. Red circle indicates the target metabolite whereas red arrow indicates where the target metabolite should be found.

Quinone formation metabolite

This biotransformation was identified with m/z 285.1479, mainly observed at RT 10.38 min and with high background interference (Fig.3.8). It was identified as the quinone formation of the parent molecule $C_{18}H_{20}O_3$. Quinone is formed in the camphor moiety by conversion of an even number of number of $-CH=$ groups into $-C(=O)$ groups with any necessary rearrangement of double bonds (IUPAC, 1997).

At RT 10.38 min, the metabolite of quinone formation was found in the simultaneous extraction samples but not in the SPLE samples. The molecule m/z 311.25 was identified as $C_{19}H_{34}O_3$ and the molecule m/z 285.2787 found in SPLE samples was identified as $C_{18}H_{37}O_2$, ethyl palmitate (fig.3.8)

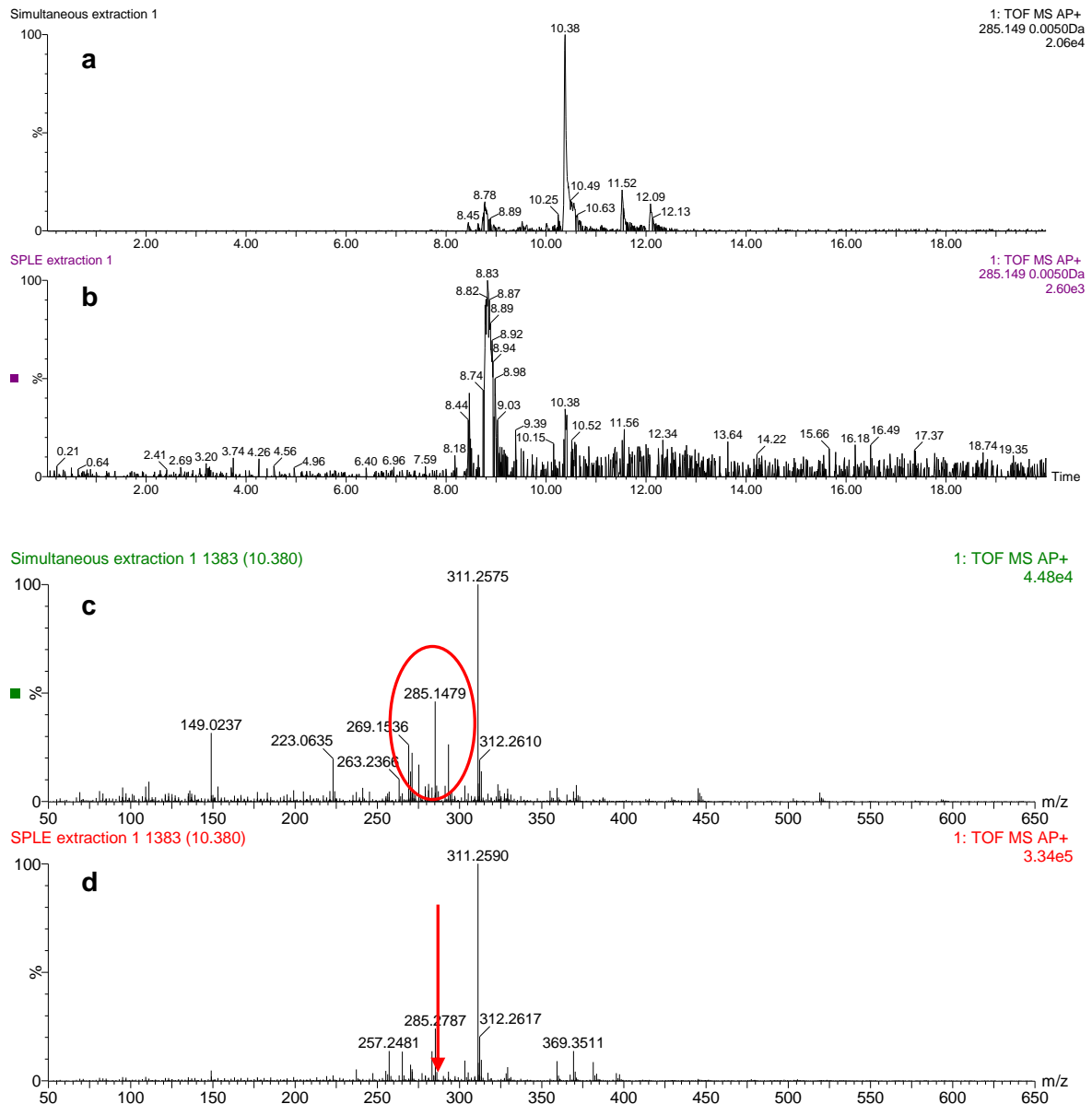


Fig. 3.8: Spectrum of simultaneous extraction (a) and SPLE (b) and chromatogram of simultaneous extraction (c) and SPLE (d) of quinone formation metabolite of 4-MBC . Red circle indicates the target metabolite whereas red arrow indicates where the target metabolite should be found.

Quinone formation + methylation metabolite

At the RT 11.30 min. and with m/z 299.1645 the quinone formation + methylation metabolite was identified. This biotransformation consists in the addition of a methyl group after the formation of the quinone explained above. Several interferences were detected in this chromatogram (fig.3.9)

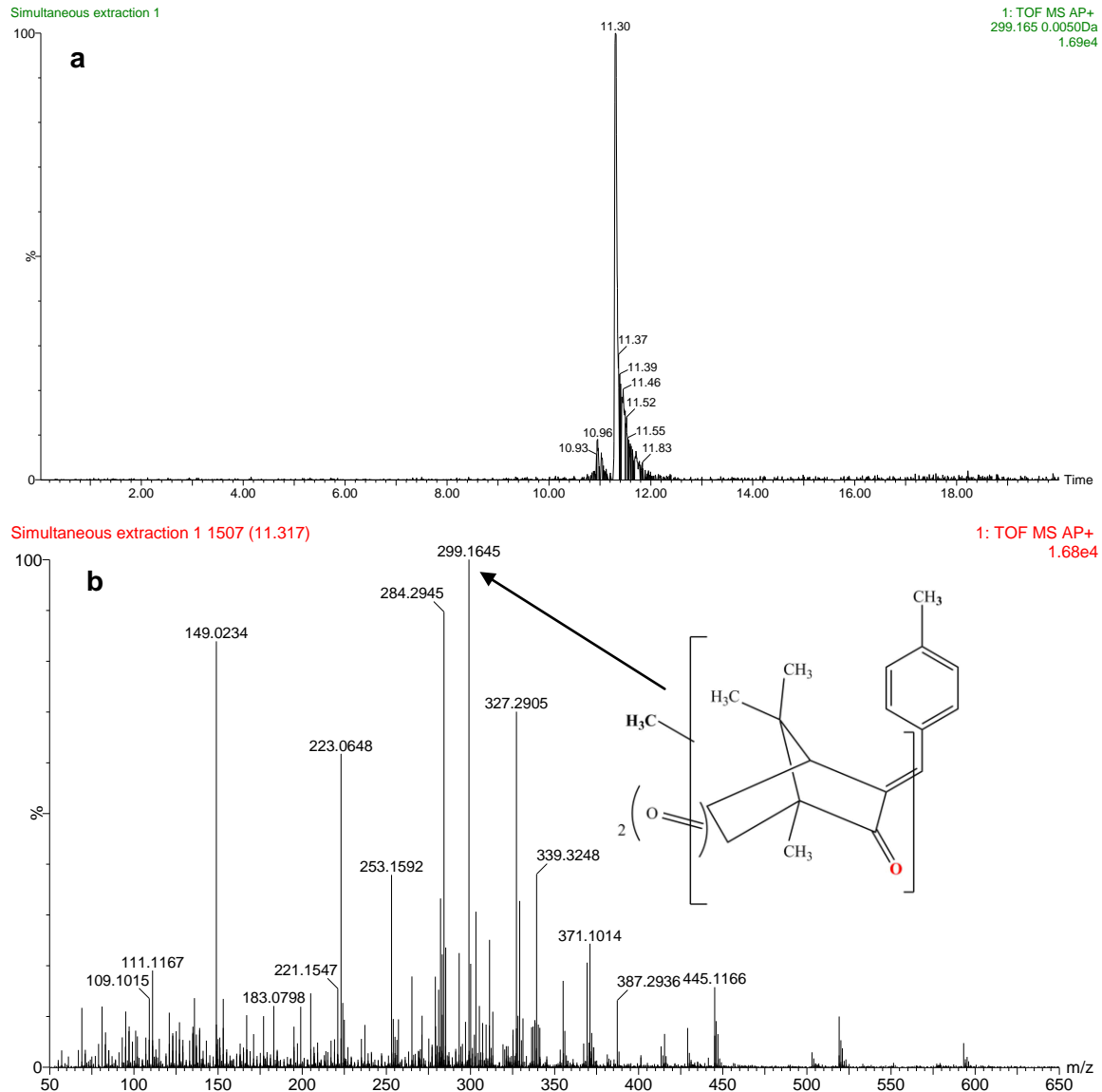


Fig. 3.9: Chromatogram (a) and spectrum (b) of quinone formation+ methylation metabolite with the proposed molecule structure in the simultaneous extraction method.

In the SPLE chromatogram samples only background interferences were found. Within the background interferences the molecule m/z 303.2319 was identified as $C_{20}H_{31}O_2$ (fig. 6.8 of annex) proposed to be a fatty acid (arachidonate or icosatetraenoate) (EMBL-EBI, 2015).

3.2. Separation and identification of metabolites of Acesulfame-K

The UPLC/MS chromatograms obtained from Manila clam tissues revealed a complex pattern of peaks showing the parent compound, acesulfame-K ($C_4H_4NO_4S$) at RT 1.80 min (fig. 3.10) with m/z 161.9861. Although the parent compound was observed in the negative ionization mode, the search and identification of metabolites were performed in both positive and negative ionization modes. ACE was present in low abundance in the exposed samples and it was not observed in the control samples.

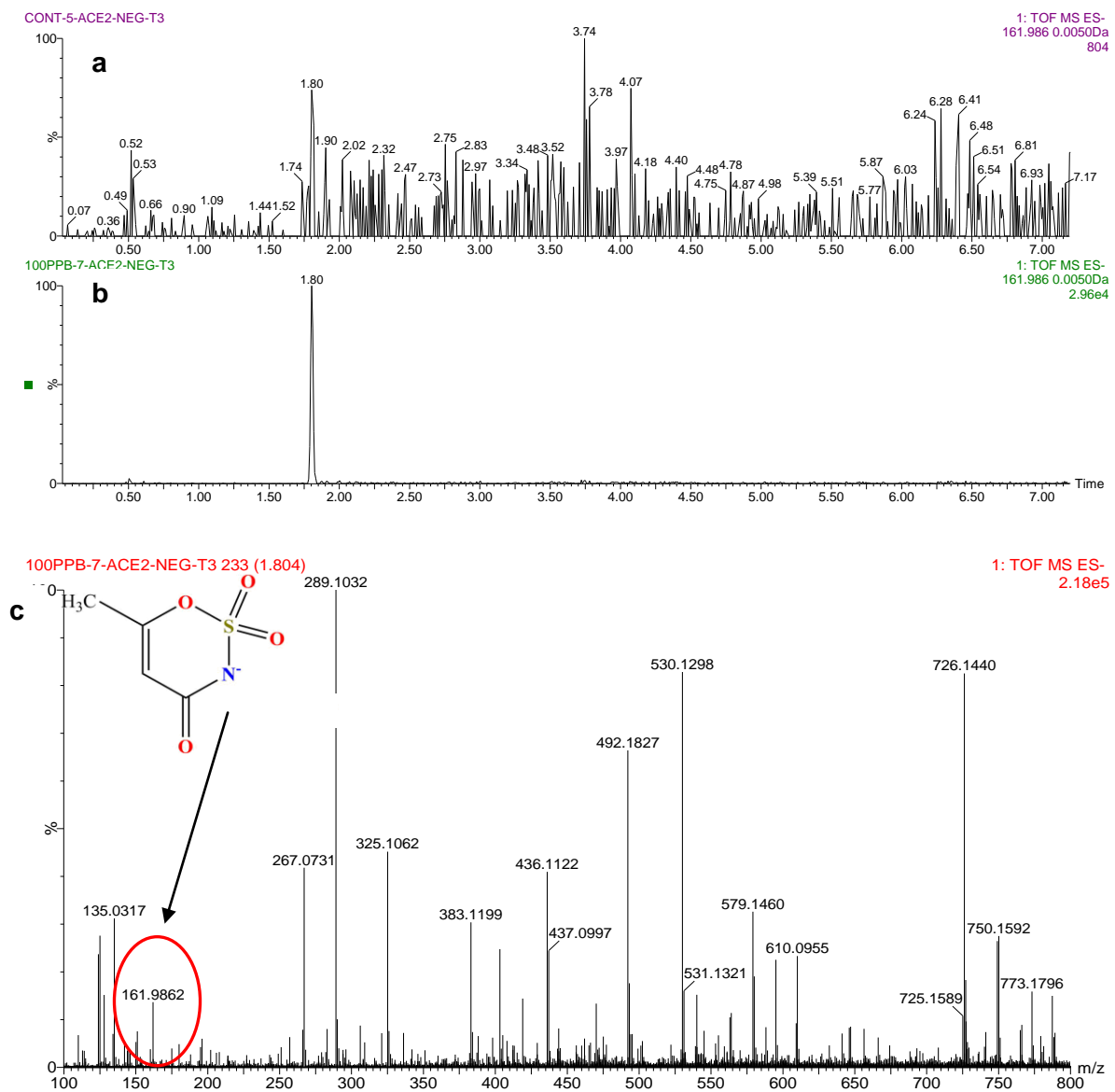


Fig. 3.10: Chromatograms of the parent compound (ACE) in control sample (a) and the exposed sample (b) followed by the spectrum of the parent compound ($C_4H_4NO_4S$) (c) in exposed samples and its molecular structure. Red circle indicates the target compound.

The post-acquisition data of the analysis of Manila clam tissues were processed with Metabolynx™ in the same ways as the samples exposed to 4-MBC. The key parameters were carefully set and a new list of possible biotransformation products was elaborated according to the physicochemical properties of the parent compound and bibliography and introduced in the software. However, only 1 possible biotransformation product was observed. This molecule was m/z 174.9569 at RT 0.5 min (fig.3.11). It was identified as the hydroxylation + desaturation metabolite of the parent compound with the formula C₄H₂NO₅S. Nevertheless, this metabolite was only identified in two of the three triplicates.

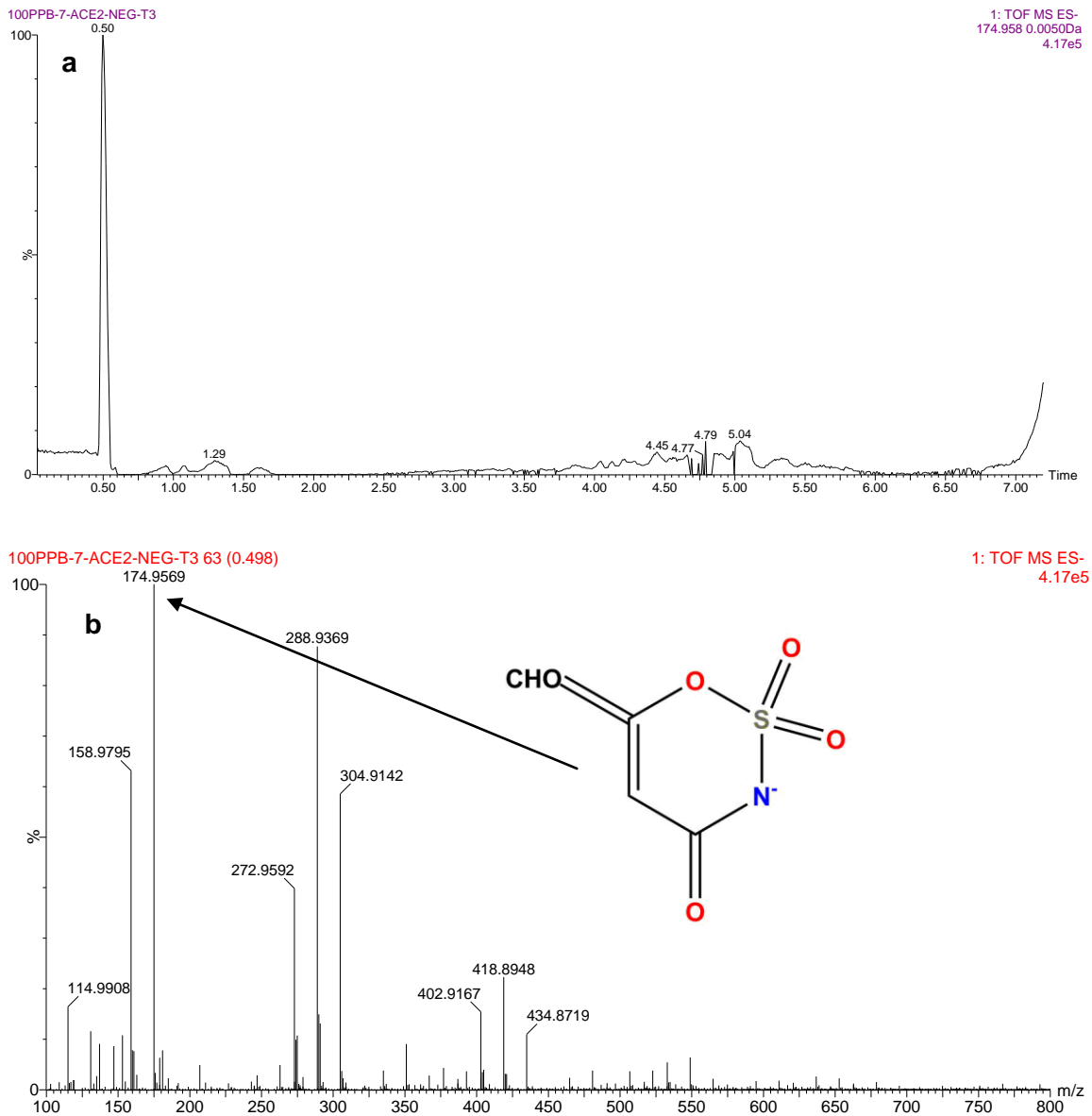


Fig.3.11: Chromatogram (a) and spectrum (b) of hydroxylation + desaturation metabolite of ACE with the proposed molecule structure.

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CHAPTER 4

Discussion

4. Discussion

Although several studies have been performed on the occurrence and possible bioaccumulation of UV filters in the environment and in organisms (Balmer et al., 2005, 2005, Benedé et al., 2014a, 2014b; Fent et al., 2010; Gago-Ferrero et al., 2015; Giokas et al., 2007; Sánchez-Quiles and Tovar-Sánchez, 2015; Tovar-Sánchez et al., 2013) and occurrence of artificial sweeteners in ground and surface waters (Bergamo et al., 2011; Gan et al., 2013; Tran et al., 2014; Van Stempvoort et al., 2011) very little is known about the metabolization and further excretion of these emerging pollutants and food additives by the organisms exposed to them (Díaz-Cruz et al., 2008)

As an attempt to fill this gap the current work presents the identification and structural characterization of the transformation products of 4-MBC and ACE by Manila clam.

In the present work the organism analysed were exposed to $100\mu\text{gL}^{-1}$ of the selected pollutants during 7 days. This concentration is higher than that found in the environment. However, if metabolites' identification analysis is wanted to be performed, higher concentrations are needed. That is, because transformation products are usually released at considerably lower concentrations than the parent compound (Badia-Fabregat et al., 2012)

4.1. Identification of 4-MBC metabolites and comparison of extraction methods.

According to the amount of 4-MBC accumulated in Manila clam tissue (729810 ng g^{-1}) and the bioconcentration factor calculated for the UV filter in the present experiment (58168 L kg^{-1}) (Tonini, 2017), it can be asserted that this compound tends to be absorbed and accumulated in the body to be metabolised and further excreted. Therefore, it is necessary to identify and characterize the new compounds formed during the transformation processes and to further assess the potential toxicity or bioaccumulation capacity, not only of parent compounds, but also of the degradation products formed (Vicent et al., 2013)

The results of this study, previously explained, show that 4-MBC is metabolized in Manila clam. Four different metabolic pathways are proposed for biotransformation of 4-MBC mediated by phase I and II enzymes (fig 4.1). The first step in the metabolic sequence results in $C_{18}H_{22}O_2$ and $C_{18}H_{22}O_3$ likely occurring by hydroxylation and double hydroxylation, respectively, of the aromatic moiety by cytochrome P450. These results agree with previous studies about identification of metabolites of 4-MBC in rats (Völkel et al., 2006) and fungus (Badia-Fabregat et al., 2012; Vicent et al., 2013) suggesting that bivalves follow a similar detoxification pathway than those described in mammals and fungi.

Additionally reduction of the parent compound leading to $C_{18}H_{24}O$ was also identified probably produced by cytochrome P450 reductase or NADPH (Richard et al., 2016).

Another biotransformation mediated by the super family of enzymes cytochrome P450 is the desaturation of the already hydroxylated compound (Fig.4.1). This process is less common and frequent for xenobiotic metabolism, however it can also be involve in the excretion of xenobiotics leading to oxidized products from the parent compound (Reilly and Yost, 2006). The process of desaturation has also been identified in fungus for xenobiotic detoxification and/or degradation by cytochrome P450 (Črešnar and Petrič, 2011; van den Brink et al., 1998)

The quinone formation is proposed as another step of metabolization. Several natural products, endogenous biochemicals and drugs contain a quinone-moiety, however they are also generated through the metabolism of organic compounds (Bolton and Dunlap, 2017; Cnubben et al., 2001) The formation of quinones by metabolic oxidation is performed by cytochrome P450 and peroxides. Quinone formation can occur in one or two metabolic steps, where the final step is usually the co-reduction of a cytochrome P450's catalytic enzyme by the substrate, thereby dehydrogenating and oxidizing the substrate into a quinone species (Hughes and Swamidass, 2017).

These phase I metabolites can suffer additional transformation by conjugation reactions in order to make them more hydrophilic making excretion easier or deactivating the biological activity of the metabolite (Hampel et al., 2016; Prakash et al., 2007). This is what happens in the next step of the metabolization process of 4-

MBC in Manila clam. The already formed quinoid metabolite is conjugated with a methyl group (-CH₃) by a phase II enzyme. This conjugation process has been also identified in the fungus *Trametes versicolor* for the excretion and reduction of toxicity of triclosan (Hundt et al., 2000) and in Sprague-Dawley rats for the metabolization and excretion of hyperoxide, a pharmacological component with antidepressant, antiviral, antifungal and anti-inflammatory activity (Guo et al., 2011). The conjugation process, part of the phase II of metabolism is also quite important as generally they result in a decrease of the toxicity of xenobiotics (Vicent et al., 2013). Although methylation reduces the polarity and hydrophilicity of the substrate, its conjugation is very important as its purpose is to deactivate the biological activity of the previously quinoid metabolite and thereby reduce its toxicity (Godin and Crooks, 1989).

All the processes of biotransformation are conducted through the production of reactive oxygen species (ROS) probably mediated by cytochrome p-450. Once the xenobiotic enters the organism a cascade of biotransformation occurs (fig 4.1.).

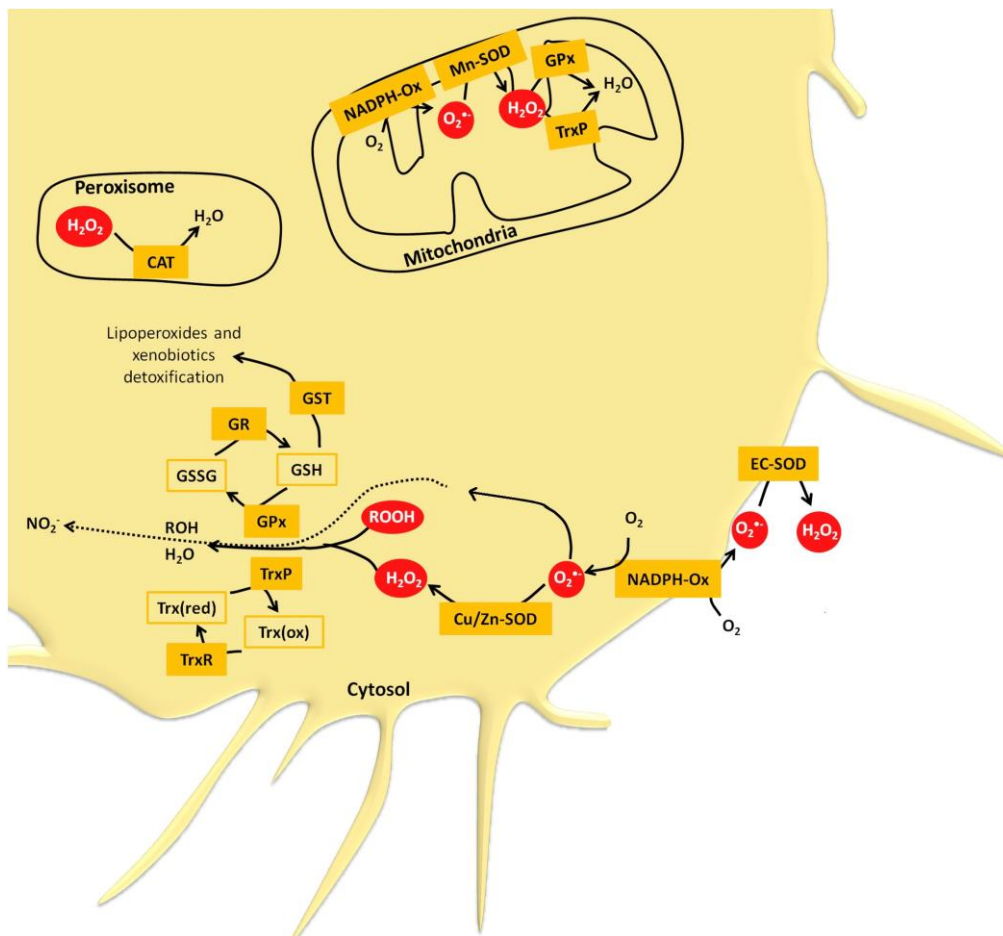


Fig. 4.1: Commonly accepted enzymatic antioxidant pathways involved in reactive oxygen species production and detoxification in bivalve molluscs. Modified from Richard et al., 2016.

When ROS are produced, oxidative stress can result in the organism, which is normally prevented by antioxidant enzymes such as superoxide dismutase (SOD), methyl transferase or glutathione-S-transferase (GST) (Prakash et al., 2007). This last one is directly involved in xenobiotics detoxification by its conjugation as it has been explained above (Hampel et al., 2016; Richard et al., 2016).

Figure 4.2 summarises the proposed biotransformation pathways of 4-MBC in Manila clam. Hydroxylation, double hydroxylation and reduction biotransformation have been proposed to occur in the aromatic ring. These results agree with previous studies that have found hydroxylation and double hydroxylation of 4-MBC as intermediate metabolizations in different organism (mammals and fungus) attached to the phenyl ring (Badia-Fabregat et al., 2012; Chisvert et al., 2012; Colipa, 2008; Janjua et al., 2004; León-González et al., 2013; Schauer et al., 2006; Tarazona et al., 2015; Vicent et al., 2013; Völkel et al., 2006)

To the best of our knowledge the quinone formation has not been previously identified as a biotransformation of 4-MBC, although it has been previously observed as a detoxification pathway for organic pollutants such as polycyclic aromatic compounds (PAH) in fungus (Sutherland, 1992) and fish (Varanasi, 1989). Thereby in order to propose a possible position for the quinone formation in the 4-MBC molecule a simulation was performed with XenoSite <http://swami.wustl.edu/xenosite/>, a computational model that predicts the specific position of the quinone formation (Hughes and Swamidass, 2017).

According to the information obtained from the computational model XenoSite, the quinone formation was proposed in the camphor moiety (fig.4.2). Nevertheless it remains clear that structural elucidation by nuclear magnetic resonance (NMR) or infrared spectroscopy would be necessary in order to certify its position. Additionally, MS/MS fragmentation spectra of the target molecule is an essential tool for assisting in molecule structural characterization (Shen et al., 2010).

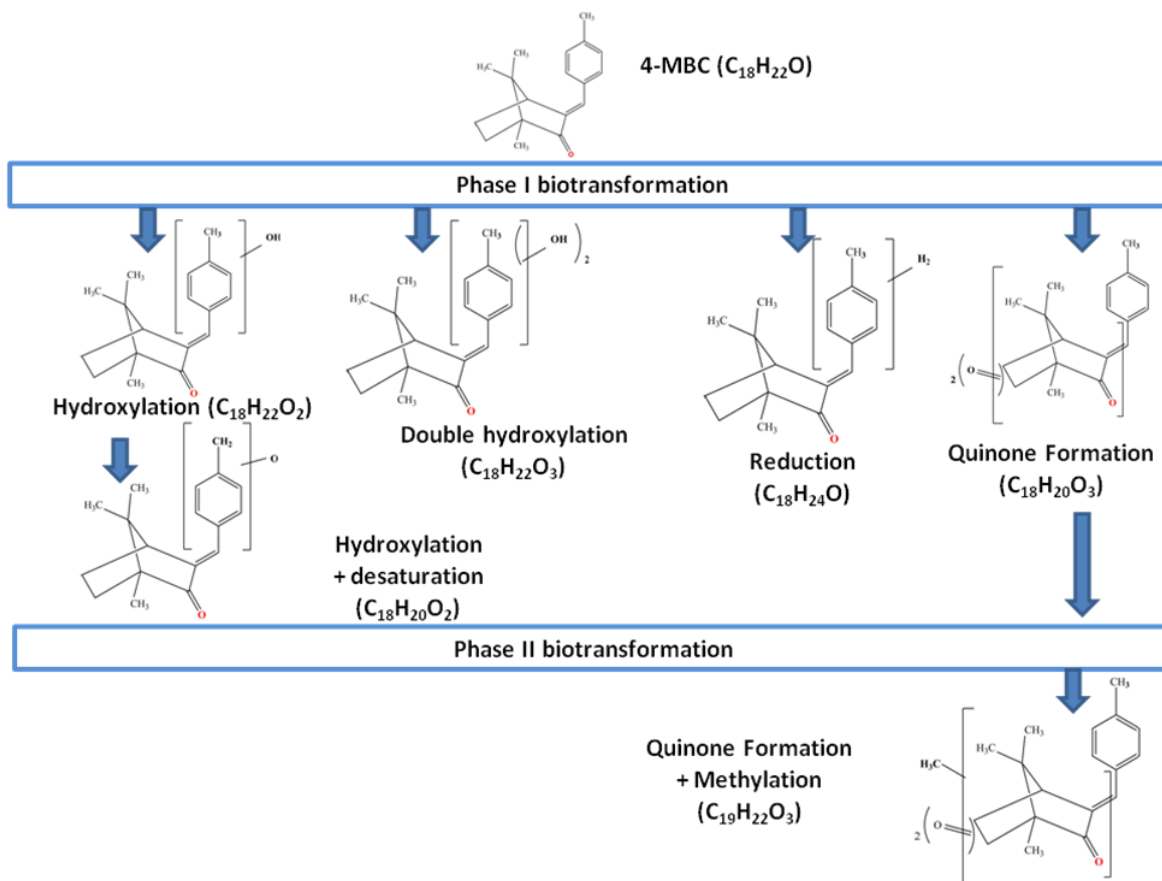


Fig. 4.2: Biotransformation pathways of 4-MBC (C₁₈H₂₂O) in Manila clam.

In relation to the comparison of the two extraction methods proposed in the present work (SPLE and simultaneous extraction) table 3.2 of the result section show the differences in the abundance of the identified peaks. Except for the reduction metabolite, in all the other cases it can be seen that the intensity of the peaks is higher with the simultaneous extraction method than with the SPLE method. Additionally, high levels of background noise were detected with the SPLE methods that do not seem to appear in the simultaneous extraction method (fig.4.3). As the intensity of the organic parent compound is very similar in both extraction methods it can be stated that the simultaneous extraction methods is more selective with the disassociation of target analytes from other cell components and seems to facilitate the detectability of metabolites (Valledor et al., 2014; Vorreiter et al., 2016). Both methods are based on the use of organic solvent extracts but SPLE uses high pressures and temperatures, that allow to take the analytes beyond their boiling point, while the simultaneous extraction method is performed at 4°C or below during

the whole process (Weckwerth et al., 2004). This difference of temperature and pressure in the extraction procedure can lead in higher extraction levels in the SPLE methods not only for the target analytes but also for interferences (such as lipids and other cell wall compounds) producing high levels of back ground noise in the chromatograms (Fig. 4.3). Nevertheless the SPLE method has showed excellent results for the simultaneous extraction of multi-class contaminants in different matrixes (Choi et al., 2014; Ghosh et al., 2011; Lund et al., 2009)

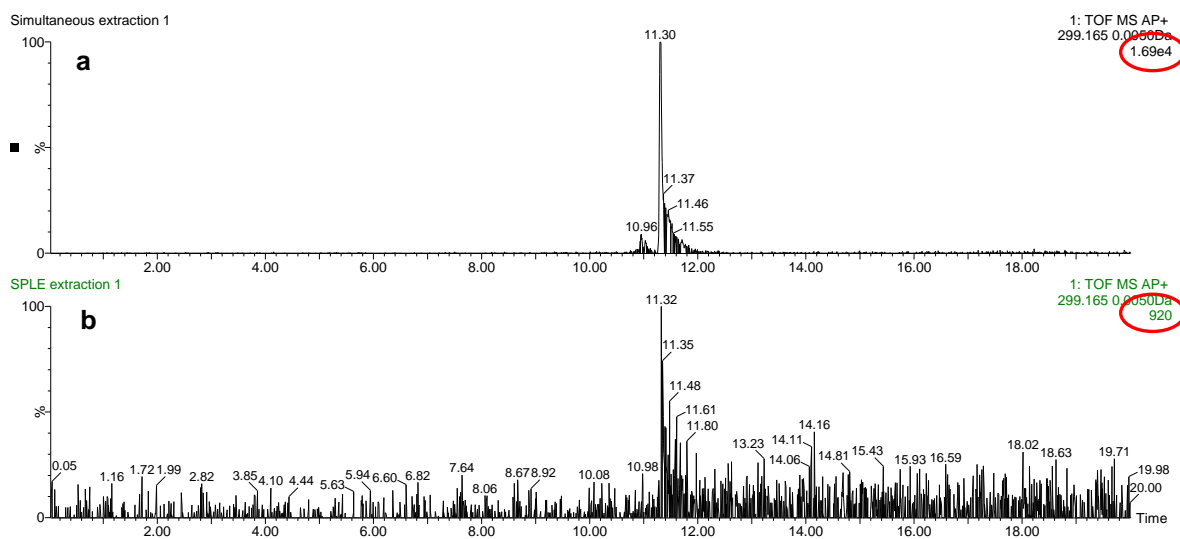


Fig. 4.3: Example of the background noise and the intensity (red circle) of the target analyte in both extraction methods: (a) Simultaneous extraction, (b) SPLE. This chromatogram is for the quinone formation + methylation metabolite of 4-MBC ($C_{19}H_{22}O_3$; m/z 299.1645).

4.2. Identification of Acesulfame-K metabolites.

Contrary to 4-MBC, ACE-K is a very water soluble compound. The amount of ACE-K accumulated in Manila clam tissue at the end of the exposure experiment was 460 ng g^{-1} and the bioconcentration factor was 0.09 L kg^{-1} (Tonini, 2017). This suggests that this food additive does not tend to accumulate and it is easily excreted. The results of this study, previously explained shows that only one metabolite of ACE-K was found in Manila clam tissues. The biotransformation identified was a hydroxylation + desaturation of the parent compound ($C_4H_2NO_5S$). This biotransformation is common in detoxification pathways, as explained above,

however, as it was not identified in the three triplicates in significant amounts, no explanation could be proposed for a biotransformation pathway.

Previous studies performed in humans about the metabolization of ACE-K (Cantarelli et al., 2009; Pierini et al., 2013; Shankar et al., 2013; Whitehouse et al., 2008) confirm that this sweetener is excreted by the kidneys after it passes through the body unchanged. Other studies with ¹⁴C-labeled ACE-K were carried out in rats, dogs and pigs with exactly the same results and no activity attributable to metabolites identified (Nabors, 2004). In microorganisms such as bacteria, no signals of metabolization of ACE-K were identified either (Brown and Best, 1988; Nabors, 2004).

In fact, due to its frequent detection and persistence ACE-K has been proposed as an ideal anthropogenic marker for wastewater in the environment by several authors (Buerge et al., 2009; Gan et al., 2013; Nödler et al., 2016; Tran et al., 2014; Van Stempvoort et al., 2011)

Nevertheless, its use as anthropogenic markers for municipal wastewater impact is only valid on a regional or national scale and within a certain time (Lange et al., 2012). This is because transformation of ACE-K in water can occur due to photodegradation (Gan et al., 2014; Li et al., 2016; Sang et al., 2014; Scheurer et al., 2014) or oxidative transformation by oxidants/disinfectants such as ozone (Scheurer et al., 2012) or permanganate, a recently commonly used oxidant in WWTPs for emerging organic pollutants (Yin et al., 2017)

Although most of the available literature on ACE-K degradation indicates that biological elimination of this artificial sweetener is not feasible, a recent study published by Castronovo et al. (2017) found that the degradation of ACE in activated sludge by microbial activity in municipal WWTPs is not as rare as previously expected. They found 5 different biotransformation products of ACE, identifying the two most abundant metabolites as sulfamic acid (SA) and acetoacetamide-N-sulfonic acid (ANSA). These results reveal a new area of research that requires further investigation.

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CHAPTER 5

Conclusion

5. Conclusion and application of the work.

In this report, the UPLC-GC/QTOFMS was used to identify possible metabolites of two emerging pollutants, the artificial sweetener acesulfame-K and the UV filter 4-MBC by the Manila clam *Ruditapes philippinarum*.

After acquisition of the data, they were processed with MetaboLynx™ software. A total of 6 different metabolites from 4-MBC compound were identified in Manila clam tissues while no significant metabolites were identified for ACE-K compound. Results showed that 4-MBC is accumulated in Manila clam tissues and metabolised to facilitate its excretion while ACE-K is almost not accumulated and excreted unchanged. These results can be explained due to the physicochemical properties of the compounds. The UV filter presents low polarity and high hydrophobicity ($\text{Log}_{\text{Kow}} = 5.92$) while the sweetener is a very polar compound that easily dissolves in water and with a very low Log_{Kow} (-1.33)

This very initial step in the identification of biotransformation products provides an example of a useful tool in the biomonitoring in ecotoxicity, as the detoxification pathways can be predicted based on the chemical structure of the molecule rather than the organism. Additionally it provides information about the behaviour of two different EP in a marine species with high economically and commercial value.

Nevertheless, it remains clear that further investigation needs to be performed in order to characterize the new compounds formed during the transformation processes and to further assess the potential toxicity or bioaccumulation capacity, not only of parent compounds. Also investigate whether the exposure of Manila clam to these pollutants and their biotransformation products can have effects on the endogenous metabolism of the organism remains essential in order to establish the mode of actions of the analysed compounds. Nevertheless once this mode of actions will be determined, they will be applicable to human health and environmental risk assessment providing scientific support for the future chemical risk management.

Annexes

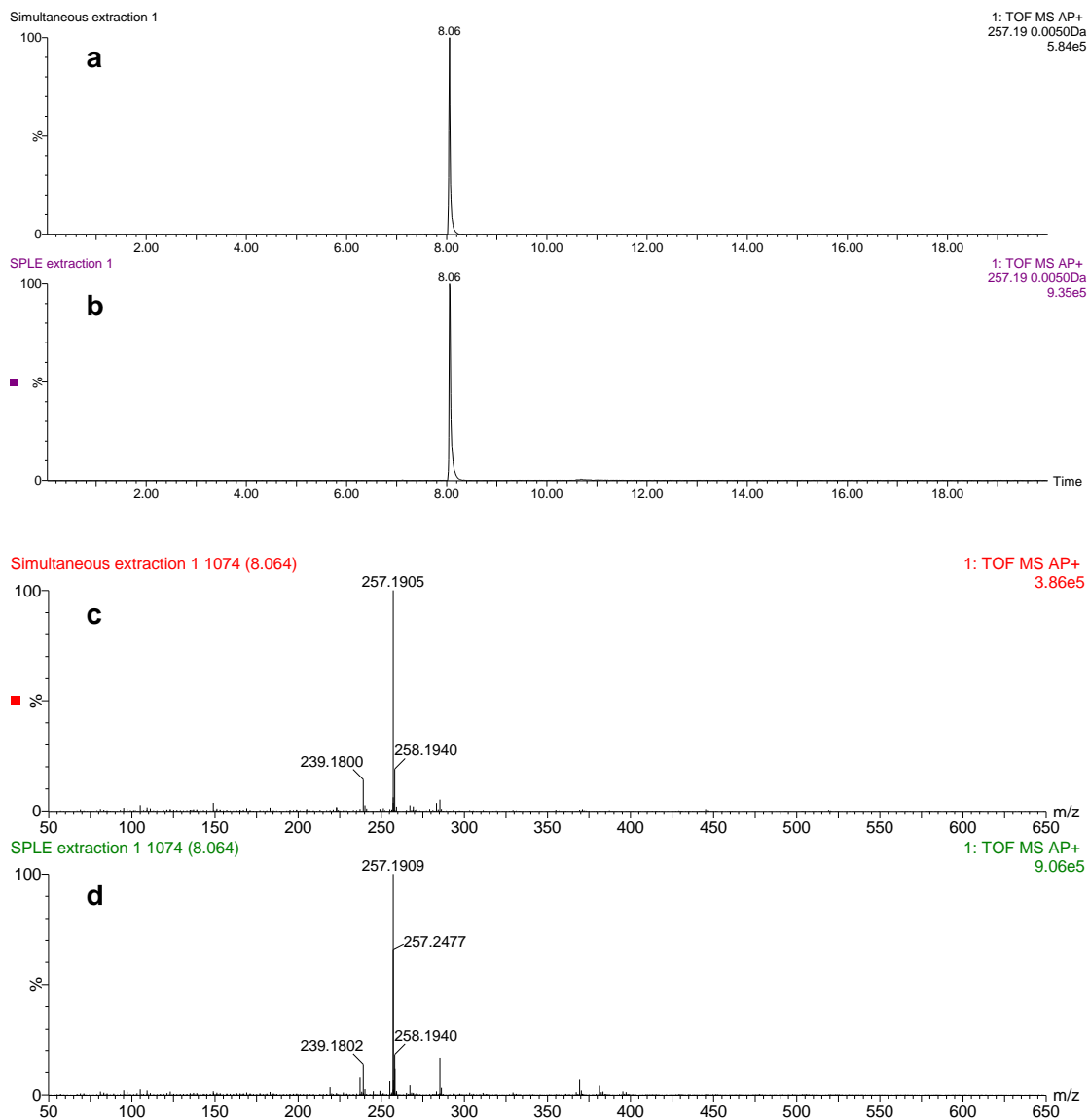


Fig. 6.1: Chromatogram of simultaneous extraction (a) and SPLE (b) and spectrum of simultaneous extraction (c) and SPLE (d) of reduction metabolite of 4-MBC.

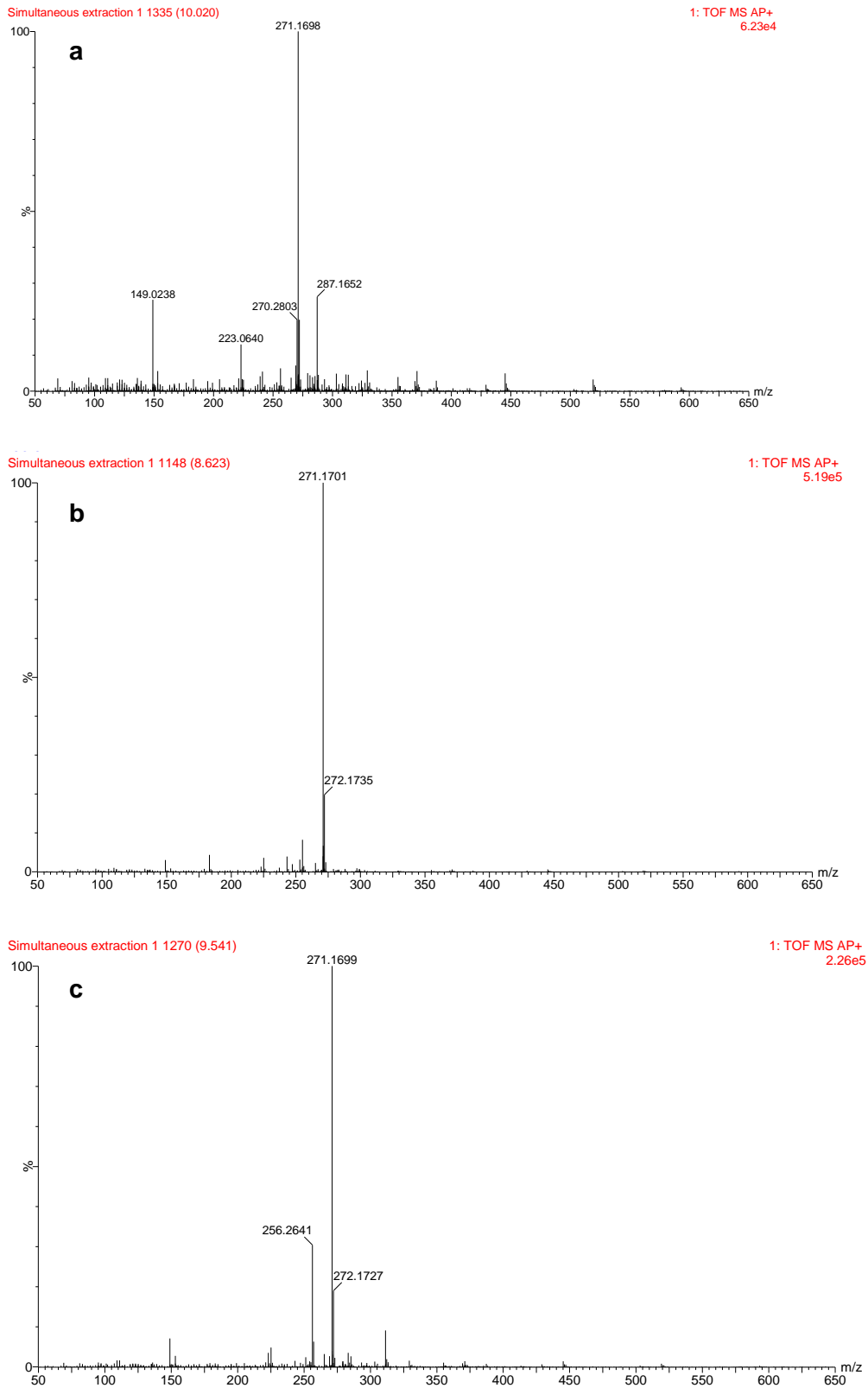


Fig.6.2.: spectrums of hydroxylation metabolite (m/z 271) at different RTs: (a) 10 min; (b) 8.62 min; (c) 9.54 min.

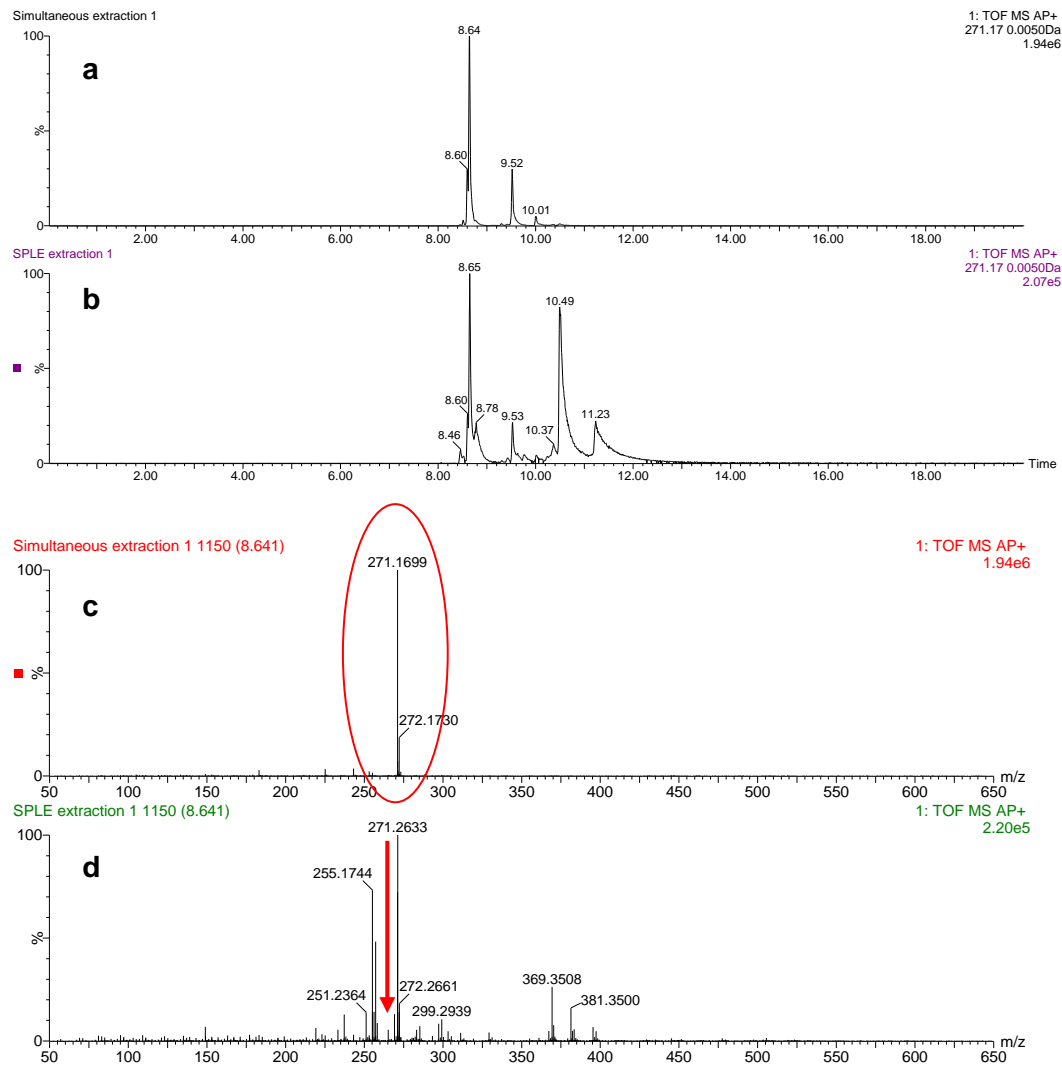


Fig. 6.3: Chromatogram of hydroxylation metabolite of 4-MBC with simultaneous extraction (a) and SPLE (b). Spectrum of hydroxylation metabolite of 4-MBC with simultaneous extraction (c) and SPLE (d). Red circle indicates the target metabolite whereas red arrow indicates where the target metabolite should be found.

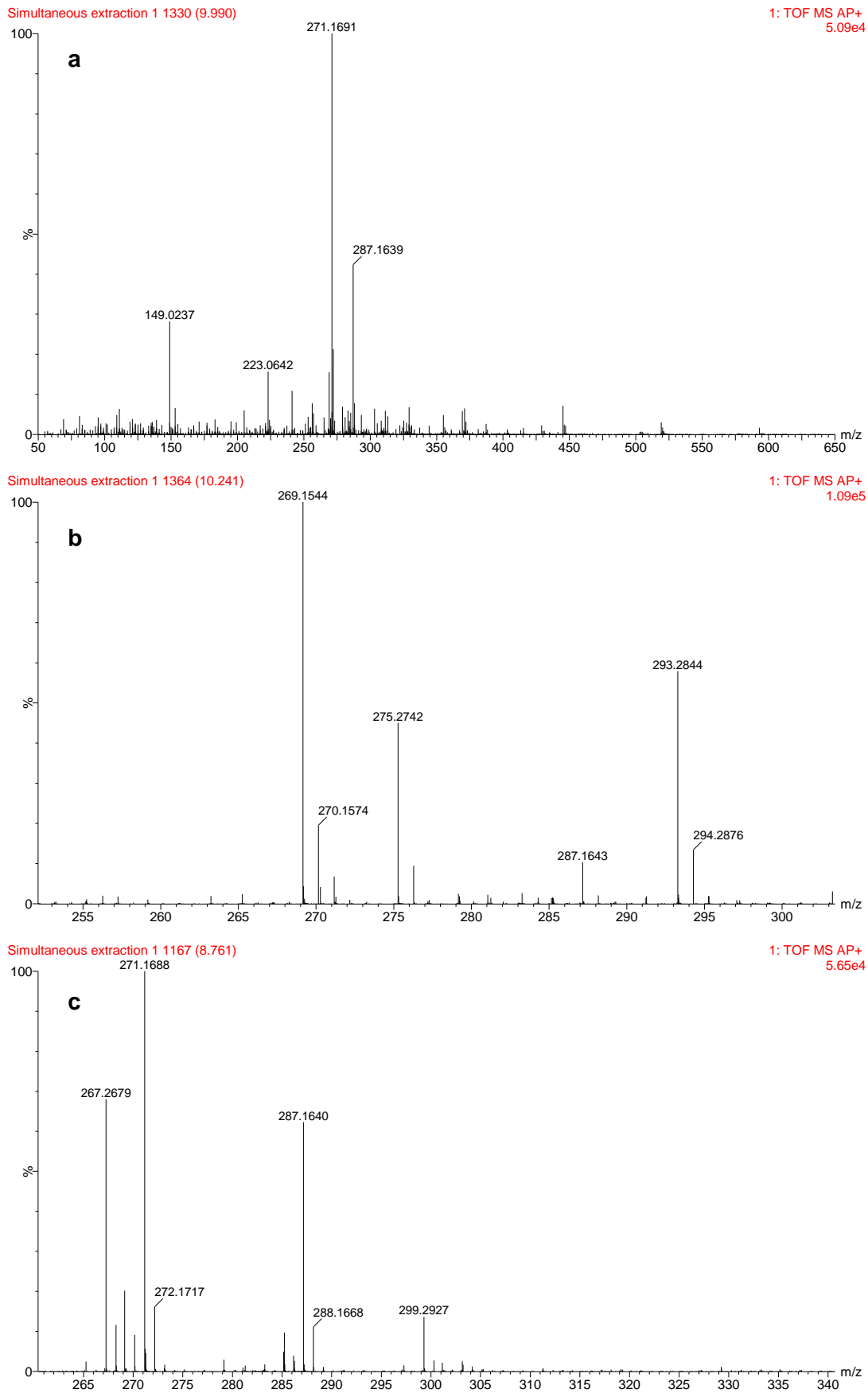


Fig.6.4 : chromatogram and spectrum of double hydroxylation metabolite at different RTs: (a) 9.9min; (b) 10.24 min; (c) 8.76 min. Red circle indicates the target motabolite

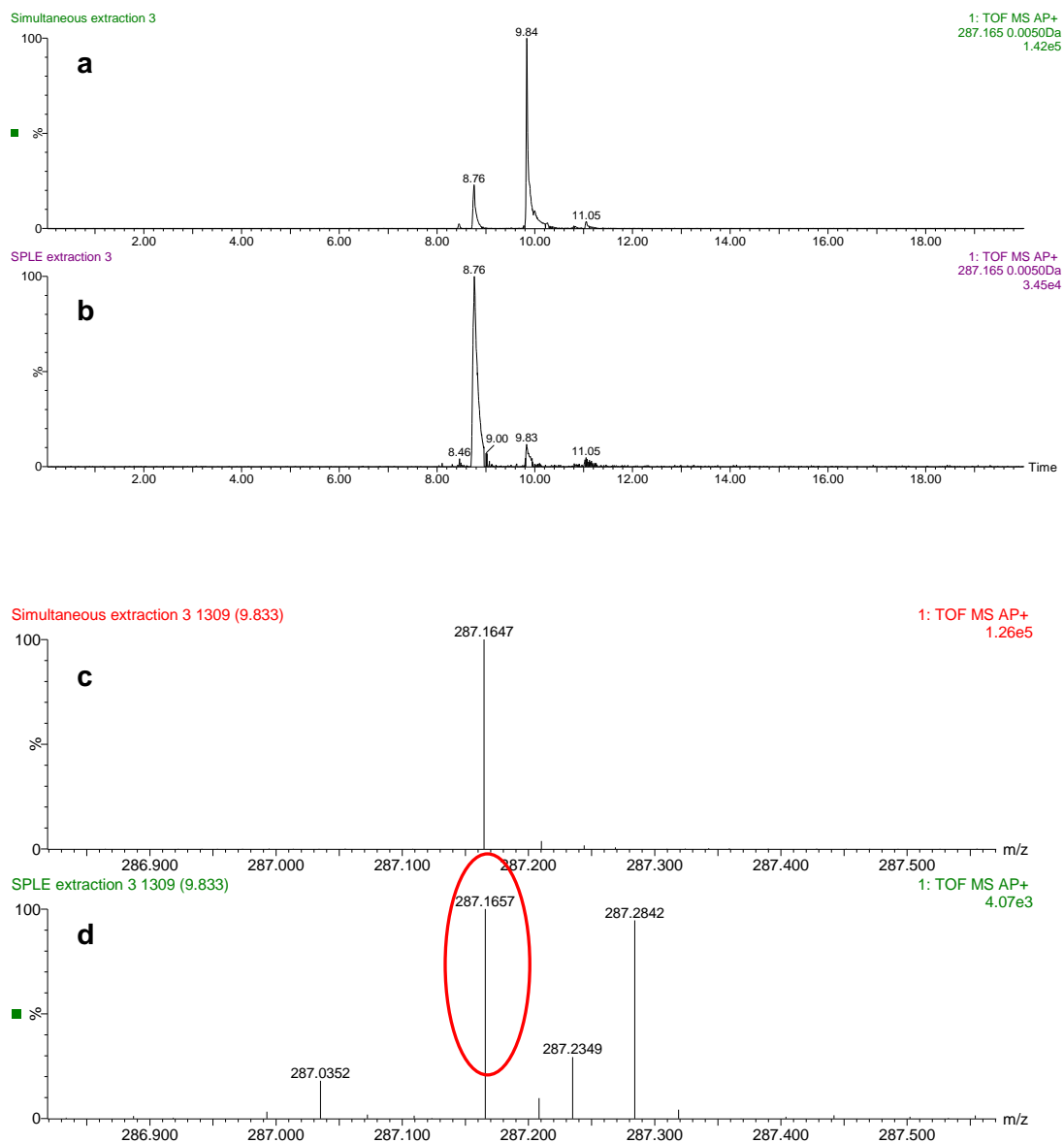


Fig. 6.5: Chromatogram of simultaneous extraction (a) and SPLE (b) of double hydroxylation metabolite of 4-MBC. Spectrum of simultaneous extraction (c) and SPLE (d) of double hydroxylation metabolite of 4-MBC . Red circle indicates the target metabolite.

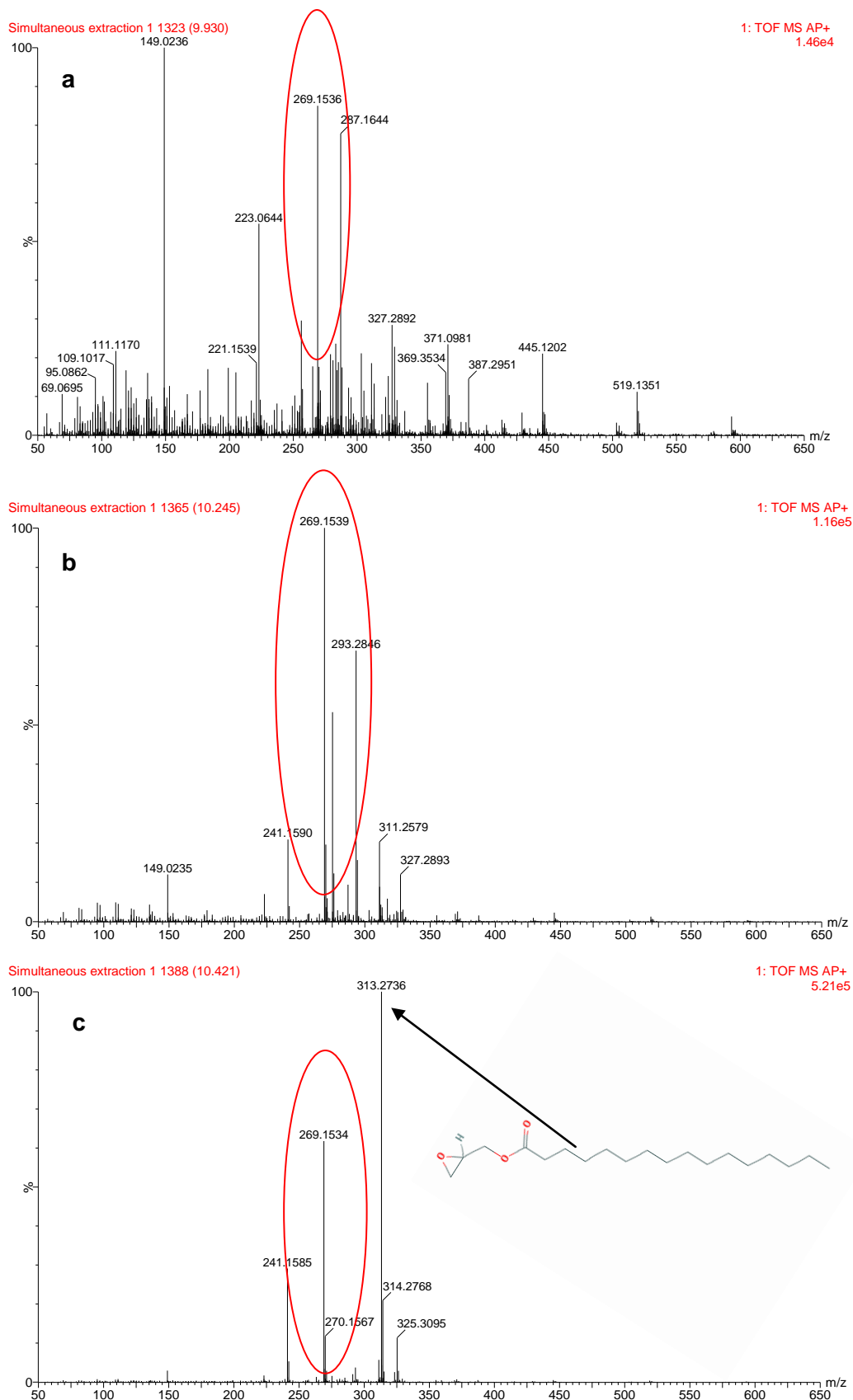


Fig 6.6: Spectrum of hydroxylation + desaturation metabolite with the proposed molecule structure at different RTs: (a) 9.9 min; (b) 10.24 min; (c) 10.42min. Red circle indicates the target motabolite

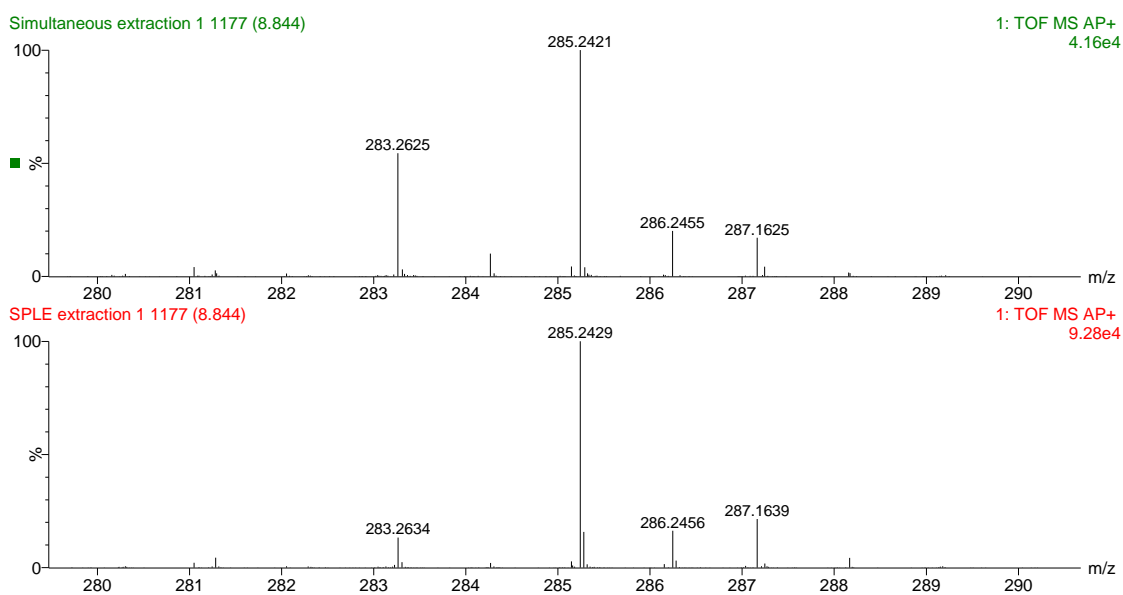


Fig. 6.7: Interferences of the molecule $C_{17}H_{33}O_3$ (m/z 285.2429) in the quinone metabolite spectrum in both methods.

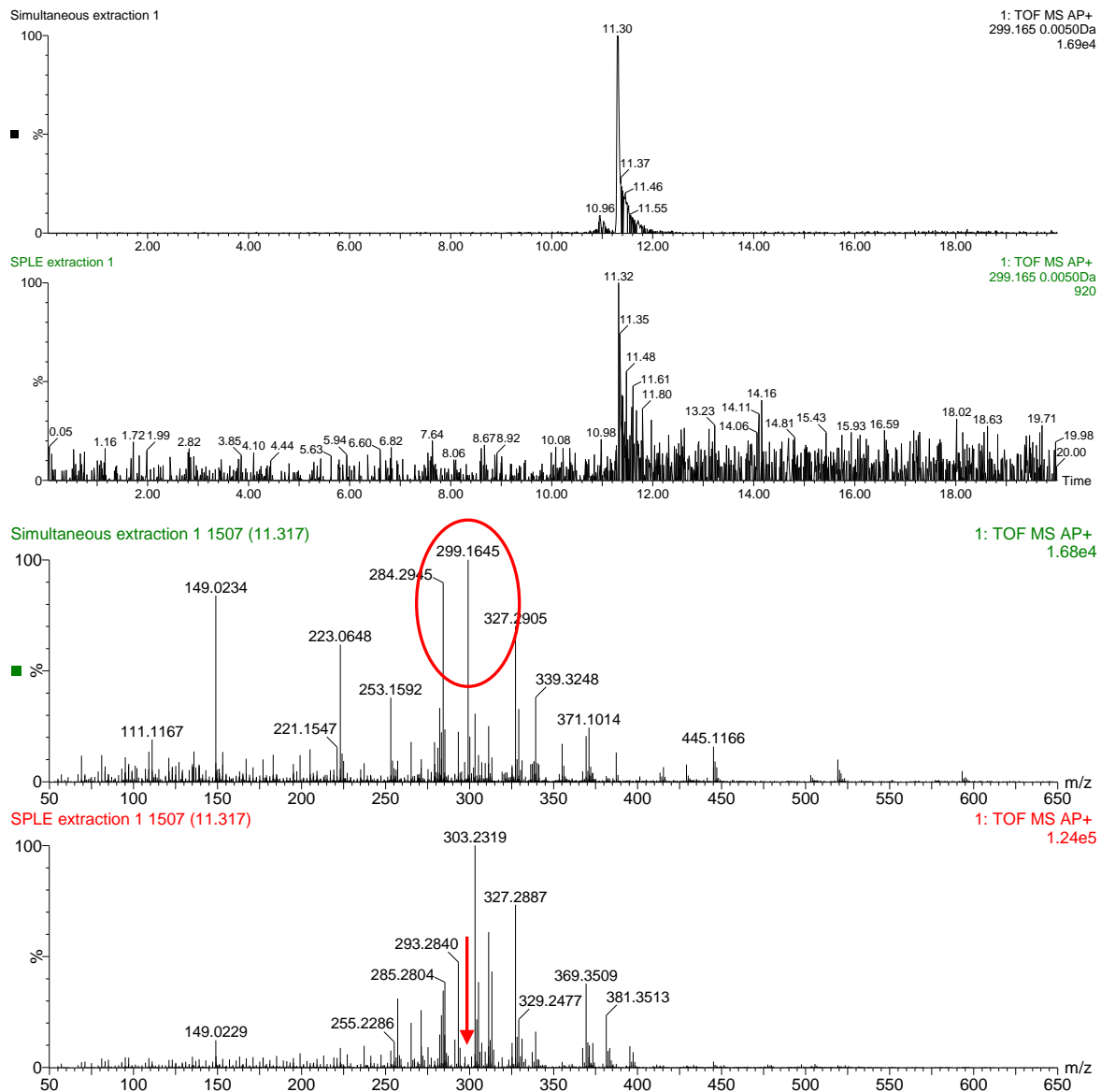


Fig. 6.8: Spectrum and chromatogram of quinone formation + methylation metabolite of 4-MBC with the two extraction methods. Red circle indicates the target metabolite whereas red arrow indicates where the target metabolite should be found.