UNIVERSIDADE DE SÃO PAULO
INSTITUTO DE QUÍMICA DE SÃO CARLOS

LETÍCIA APARECIDA MARQUES

ANALYTICAL DEVELOPMENTS FOR ORGANOCHLORINATED COMPOUNDS DETERMINATION IN MILK

TESE DE DOUTORADO

SÃO CARLOS
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Thesis submitted to São Carlos Institute of Chemistry from Universidade de São Paulo as part of the requirements for the award of the degree of doctorate in Chemistry.

Area of concentration: Analytical and Inorganic Chemistry

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SÃO CARLOS - SP
2021

Assinatura:
Data:

Ficha Catalográfica elaborada pela Seção de Referência e Atendimento ao Usuário do SBI/IQSC

Marques, Letícia Aparecida
Analytical developments for organochlorinated compounds determination in milk / Letícia Aparecida Marques. — São Carlos, 2021.
138 f.

Tese (Doutorado em Química Analítica e Inorgânica) — Instituto de Química de São Carlos / Universidade de São Paulo, 2021. Edição revisada

Orientador: Prof. Dr. Stanislau Bogusz Junior
Coorientador: Prof. Dr. Emanuel Carrilho

1. Leite. 2. PCB. 3. Pesticidas. 4. Cromatografia gasosa. 5. Microfluídica. I. Título.

Sonia Alves - CRB: 4280/8

DEDICATION

To my mother, my father and brother

You are essential to me

To those who believe that science might create a better world

ACKNOWLEDGMENTS

To Professor Dr. Stanislau Bogusz Junior for accepting me as his first Ph.D. student. To Professor Dr. Emanuel Carrilho for sharing his research experience. It was an honor to be advised by you both. Thank you for being part of this essential step of my professional career.

I would like to thank the colleagues of the Laboratório de Química da Aguardente – Renato Durello, Stephano Marques, Larissa Almeida, Pamela Grizotto, Lucas Silva, Mirella Romanelli. To the members of the Bioanalítica, Microfabricação e Separações (BioMiCS) group – Amanda Hikari, Bruno Araújo, Cleyton Makara, Fabiana Marques, Fábio Damasceno, Jéssica Albuquerque, Jéssica Feitor, Juliana Alberice, Juliane Borba, Jonathan Catai, Laís Brazaca, Letícia Jordão, Letícia Pontes, Lucas Ximenes, Luciana Capellari, Ma Hui Ling, Mariana Bortholazzi, Vinícius Acquaro, Vinícius Ferreira, Victor Takekawa, Thiago Segato, Weliton Batiston. Thank you all for the friendly environment at the lab, and study sessions! To Professor Dr. Claudimir Lucio do Lago and Dr. Kelliton José Mendonça Francisco for the construction of the homemade C^4D equipment and troubleshooting support.

To the Universidade de São Paulo (USP) and Instituto de Química de São Carlos (IQSC) for the opportunity. To the technical-administrative staff from IQSC-USP, particularly Claudia, Jennifer, and Silmara, for assisting us during the lab routine.

To the doctoral fellowship process #142056/2016-2 from the National Council for Scientific and Technological Development (CNPq). And to grant #2017/19478-9, São Paulo Research Foundation (FAPESP). In addition, this research was supported by LNNano - Brazilian Nanotechnology National Laboratory, CNPEM/MCTI. Instituto Nacional de Ciência e Tecnologia de Bioanalítica - INCTBio (FAPESP grant no. 2014/50867-3 and CNPq grant no. 465389/2014-7) is kindly acknowledged for the grants.

I am also grateful to the Ministry of Agriculture, Livestock and Supply (MAPA), especially to the Federal Laboratory of Animal and Plant Health and Inspection (LFDA) in São Paulo state, our partner in this project from 2017 to 2020. Our first contact at the LFDA was with Luís and Amanda, who were essential to the establishment of this partnership. I extend my thanks to federal auditors MSc. Carlos Juliano, and Dra. Vanessa Santos, responsible for the coordination of the Laboratório de Resíduos e Contaminantes em Alimentos (RCA) of LFDA-SP. To all collaborators from RCA lab – Dra. Adriana, Ana Claudia, Eduardo, Fernando, Dra. Eloísa de Paula (*in memorian*), Laila, Paula, Marisa, Roberto. I also would like to thank Dra. Lívia Cavaletti, MSc. Milene Berbel, Henrique Horta, Rodrigo Labello, Juliana Pereira, and

Estela Bonilha for the professional exchanges and lunches together. You taught me so much, guys. It was honored to work with you.

To my friends – Aline, Dasiane, Henrique, Victor Hugo – who I consider as true brothers. Thank you all for being at my side in the happy and difficult moments of this journey.

To my family. My mother, Maria Antonia, and my father, Sérgio, who supported me through all my life. I certainly could not develop this thesis without your encouragement and love. I also thank my only brother, Lucas, for his inspiring serenity and caring. To my grandmother, Luzia, for her tenderness and prayers.

To Neylor, my dear boyfriend. Your support was essential during the writing of this thesis. Thank you for your patience, understanding, and beloved presence during this journey.

Last but not least, I thank God for the gift of life and good health through these years.

EPIGRAPH

“Science is a way of thinking much more than it is a body of knowledge.”

Carl Sagan

RESUMO

Os bifenilos policlorados (PCBs) e pesticidas organoclorados (OCPs) foram intensamente utilizados na agricultura e indústria até os anos 1970, quando efeitos tóxicos à saúde humana causados pela exposição aos PCBs e OCPs foram observados. Desde então, legislações internacionais levaram à regulamentação ou banimento do seu uso, e também passaram a exigir o monitoramento desses compostos para verificar a contaminação em alimentos. O consumo de leite é uma das principais formas de exposição não-ocupacional aos PCBs e OCPs. Desta forma, esta tese teve o objetivo de desenvolver métodos analíticos modernos para o monitoramento de PCBs e OCPs em leite. No Capítulo 1, é apresentada uma revisão sobre as características químicas dos PCBs e OCPs, destacando-se a relevância das técnicas de preparo de amostra mais usuais para extração de PCBs e OCPs de leite; e sobre o desenvolvimento de dispositivos microfluídicos para detectar compostos organoclorados em amostras de leite. No Capítulo 2, são apresentados métodos de preparo de amostra para a extração de PCBs de amostras de leite integral ultrapasteurizado (UHT). As técnicas de microextração em fase sólida (SPME) e QuEChERS miniaturizada foram testadas e otimizadas usando planejamento experimental, seguido de análise por cromatografia gasosa acoplada à espectrometria de massas sequencial (GC-MS/MS). Apesar de a SPME ter apresentado melhor sensibilidade, optou-se por utilizar o método miniaturizado de QuEChERS para as análises subsequentes, considerando o elevado número de amostras analisadas. A QuEChERS miniaturizada mostrou ser uma alternativa viável para a extração de PCBs de leite UHT, oferecendo um reduzido custo por análise, sendo uma mais ambientalmente amigável que a QuEChERS em escala convencional. Em seguida, o Capítulo 3 apresenta a validação analítica da QuEChERS miniaturizada para 9 PCBs (congêneres 28, 52, 101, 118, 138, 153, 156, 179 e 180) de acordo com as orientações do INMETRO e SANTE/11813/2017. O método mostrou-se apropriadamente seletivo, linear (R^2 de 0,9875 a 0,9933), preciso (valores de RSD variando de 2,04 a 11.11%), e exato (recuperação de 95,81 a 112,83%). O monitoramento da contaminação de PCBs em amostras de leite UHT do estado de São Paulo foi efetuado em 81 amostras de marcas locais; nenhuma delas apresentou níveis de concentração de PCBs acima dos limites de detecção. E, no Capítulo 4 é apresentado o desenvolvimento de um microdispositivo baseado na detecção condutométrica sem contato capacitivamente acoplada (C^4D) para detecção específica de DDT através de sistema de bioreconhecimento utilizando a abordagem aptâmero-ligante. Uma microfabricação de baixo custo foi possível usando máscaras de papel adesivo, adesivo dupla-face e lamínulas de vidro como substrato. Os microdispositivos foram funcionalizados usando glutaraldeído e um aptâmero específico para DDT; a interação ligante-aptâmero promoveu uma redução significativa do sinal de C^4D, mostrando que o aptasensor pode ser empregado como dispositivo semi-quantitativo para rápida e fácil avaliação da presença de DDT em amostras de alimentos ou ambientais.

Palavras-chave: leite, pesticidas organoclorados, PCBs, GC-MS/MS, QuEChERS, microfluídica, C^4D, microfabricação de baixo custo, aptasensor.

ABSTRACT

Polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) were extensively used for agricultural and industrial purposes until the 1970s when toxicity effects to human health caused by exposure PCBs and OCPs were noticed. Since then, international laws lead to regulate or banish their usage, and also demand these compounds monitoring to verify food contamination. Milk intake is one of the main forms of non-occupational exposure to PCBs and OCPs. Therefore, this thesis aimed at developing modern analytical methods for PCBs and OCPs monitoring in milk. In Chapter 1, a review about chemical properties of PCBs and OCPs is presented, highlighting the mostly used extraction techniques for PCBs and OCPs from milk samples; and about microfluidic developments to detect organochlorinated compounds in milk samples. In Chapter 2, sample preparation for PCBs extraction from ultra-high temperature (UHT) whole-milk are presented. Solid-phase microextraction (SPME) and a miniaturized QuEChERS were tested and optimized using experimental designs, followed by analysis by gas chromatography coupled to sequential mass spectrometry (GC-MS/MS). Despite SPME method presented higher sensitivity, it was chosen to use the miniaturized QuEChERS procedure to subsequent analysis once an automatized system to perform SPME extractions and injections was not available. The miniaturized QuEChERS procedure showed a suitable alternative to PCBs extractions from UHT milk, offering a reduced cost per analysis and being considered a greener methodology. Next, Chapter 3 presents analytical validation of the miniaturized QuEChERS for 9 PCBs (congeners 28, 53, 101, 118, 138, 153, 156, 170, and 180) according to INMETRO and SANTE/11813/2017 guidelines. The method showed to be properly selective, linear (R^2 values from 0.9875 to 0.9933), precise (RSD values varying from 2.04 to 11.11%), and accurate (recovery from 95.81 to 112.83%). An assessment of PCBs contamination in UHT milk samples from São Paulo state was performed in 81 samples purchased from local brands with a positive outcome: none of them presented PCBs concentration above detection limits. In Chapter 4, the development of a capacitively coupled contactless conductivity (C^4D) detection-based microdevice is presented, with specific DDT detection through a biorecognition system using the aptamer-ligand approach. Low-cost fabrication of microdevices was feasible by utilizing paper masks, double-sided adhesives, and glass coverslips substrates. They were functionalized using glutaraldehyde and an aptamer for specific DDT detection; the aptamer-ligand interaction promoted a remarkable C^4D signal reducing, showing that the proposed aptasensor can be used as a semi-quantitative device for a fast and easy evaluation of DDT presence in food or environmental samples.

Key words: milk**,** organochlorine pesticides, PCBs, GC-MS/MS, QuEChERS, microfluidic, C^4D, low-cost microfabrication, aptasensor.

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LIST OF ABBREVIATIONS

μ-TAS - Micro-total analysis systems

AC - Alternating current

ADI - Acceptable Daily Intake

AhR - Aryl-hydrocarbon receptor

ANOVA - Analysis of variance

ANVISA - Brazilian Health Regulatory Agency

APTES - (3-Aminopropyl)triethoxysilane

BMI - Body mass index

BSA - Bovine serum albumin

C^4D - Capacitively coupled contactless conductivity

CAR - Carboxen

CCD - Central composite design

CE - Collision energy

D.f. - Degree of freedom

DL - Dioxin-like

DoE - Design of Experiments

DPV - Differential pulse voltammetry

DVB - Divinylbenzene

EC - European Commission

EDC - 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide

EDTA - Ethylenediaminetetraacetic acid

EI - Electron ionization

EIS - Electrochemical impedance spectroscopy

FAO - Food and Agricultural Organization

FTO - Fluorine-doped tin oxide

GC - Gas chromatography

GC-ECD - Gas chromatography with electron capture detector

GC-MS - Gas chromatography coupled to mass spectrometry

GC-MS/MS - Gas chromatography with sequential mass spectrometry

GPC - Gel permeation chromatography

HS-SPME - Headspace solid-phase microextraction

INMETRO - National Institute of Metrology, Quality and Technology

ISTD - Internal standard

IUPAC - International Union of Pure and Applied Chemistry

LC - Liquid chromatography

LLE - Liquid liquid extraction

LOC - Lab-on-a-chip

LOD - Limit of detection

LOQ - Limit of quantification

MAPA - Ministry of Agriculture, Livestock and Supply

MRL - Maximum residue limit

MS - Mass spectrometry

NDL - Not dioxin-like

NHS - *N*-Hydroxysuccinimide

NIR - Near-infrared

NMR - Nuclear magnetic resonance

OLS - Ordinary Least Squares

PA - Polyacrylate

PDMS - Polydimethylsiloxane

PNCRC - National Plan for Control of Residues and Contaminant

POC - Point-of-care

POP - Persistent organic pollutants

PSA - Primary-secondary amine

PTFE - Polytetrafluoroethylene

QuEChERS - Quick, Easy, Cheap, Effective, Rugged, and Safe

ROS - Reactive oxygen species

RSD - Residue standard deviation

RT - Retention time

SELEX - Systematic Evolution of Ligands by Exponential Enrichment

SERS - Surface-enhanced Raman spectroscopy

SLE - Solid-liquid extraction

SPE - Solid phase extraction

SPME - Solid-phase micro extraction

TCDD - 2,3,7,8 - tetrachlorodibenzo-*p*-dioxin

TE - tris-EDTA

TEF - Toxic equivalent factor

TEQ - Toxic equivalent quotient

TMDI - Theorical maximum daily intake

UHT - Ultra-high temperature

UNEP - United Nations Environmental Program

WHO - World Health Organization

WLS - Weighted least square

SUMMARY

CHAPTER 1 - A Review of Polychlorinated Biphenyls and Organochlorine Pesticides in Bovine and human breast milk – Chemical aspects, methods of extraction and new analysis approaches

1.1 Introduction

For many years, humans have been using molecules to improve agricultural production and prevent losses from pest infestations. Since the introduction of halogenated compounds, they had been used extensively for those purposes until scientific evidence demonstrating their hazardous effects on human health. Among these halogenated molecules, the chlorinated-based pesticides are the most relevant ones. This chapter deals with polychlorinated biphenyls and organochlorine pesticides – their chemical characteristics, toxicity, and regulations. Their presence as contaminants in human breast milk or animal milk, sample preparation techniques for these compounds, and also microfluidic approaches as new possibilities to determine those compounds in milk matrices will be discussed in the following sections.

1.1.1 PCBs - Chemical definition, terminology, and usage

Polychlorinated biphenyls (PCBs) are organochlorine compounds recognized as persistent organic pollutants (POPs). PCBs have a general molecular formula $C_{12}H_{10-n}Cl_n$ with *n* varying up to 10 atoms and might be produced from biphenyl groups' reaction with chlorine gas. There are several possible structures, regarding position and/or number of chlorine substituents in the primary biphenyl structure, which promote a range of 209 molecules, known as congeners. According to Lehmler and Robertson (2001), at least 78 congeners might be considered rotational isomers, and nineteen are atropisomers, which occur when chlorine atoms are present in two para- and in two meta- positions (LEHMLER & ROBERTSON, 2001; PESSAH *et al.*, 2009).

PCBs were first used as additives of lubricants oils, coolants, and dielectric fluids to closed systems (capacitors, transformers, machines, *etc*.) and open-ended purposes, such as plasticizers in inks and adhesives, rubbers, and resins. Estimates suggest that around 1.5 million cubic tons of commercial mixtures of PCBs were sold all over the world since the 1920s, under different names by different manufacturers, such as Aroclor (United States), produced by Monsanto companies; Phenochlor (France), made by Prodelec; Clophen (Germany), manufactured by Bayer; Kanechlor (Japan), made by Kanegafuchi Chemical Co. (GRIMM *et al.*, 2015; VOOGT, BRINKMAN, 1989).

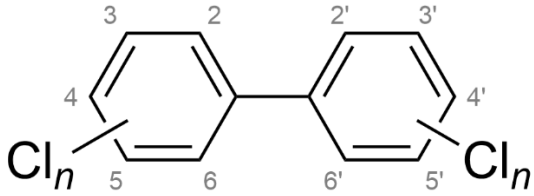
Notwithstanding their banning for over forty years, PCBs' physicochemical characteristics turn possible to find residual levels in the environment and foodstuffs. It is estimated that approximately 40% of total PCB's production remains as pollutants. PCBs have excellent chemical stability, good thermal stability, and high lipophilicity, ensuring their

biomagnification through trophic levels. Their persistence is so high that the German guideline DIN 51527 uses six PCBs congeners 28, 52, 101, 138, 153, and 180 as environmental contamination indicators (PEREIRA, 2004; COSTABEBER *et al.*, 2006).

The World Health Organization (WHO) estimates that more than 90% of human exposure occurs through diet, especially by consuming fish, milk, red meat, and dairy products. This type of indirect contact is known as non-occupational exposure and it is why many regulatory agencies or national authorities worldwide have been monitoring animal foods (WALSTRA, 2005; WHO, 2003).

PCBs are commonly named by a systematic enumeration based on BZ number, proposed initially by Ballschmiter and Zell's study (BALLSCHMITER, ZELL, 1980). It is a notation from 1 to 209 that orders congeners from mono- to decachloro-substituted molecules. The International Union of Pure and Applied Chemistry (IUPAC) uses a nomination based on chlorine atoms' relative position to biphenyls structure. First, the ring binding between aromatic rings is taken as position 1; then numeration must be increased anticlockwise for the left side ring as well as increases for the right side of aromatic ring in a clockwise with index addition as demonstrated in Figure 1 - Basic polychlorinated biphenyl structur (PENTEADO & VAZ, 2001; PEREIRA, 2004; MILLS III, THAL & BARNEY, 2007).

Figure 1 - Basic polychlorinated biphenyl structure



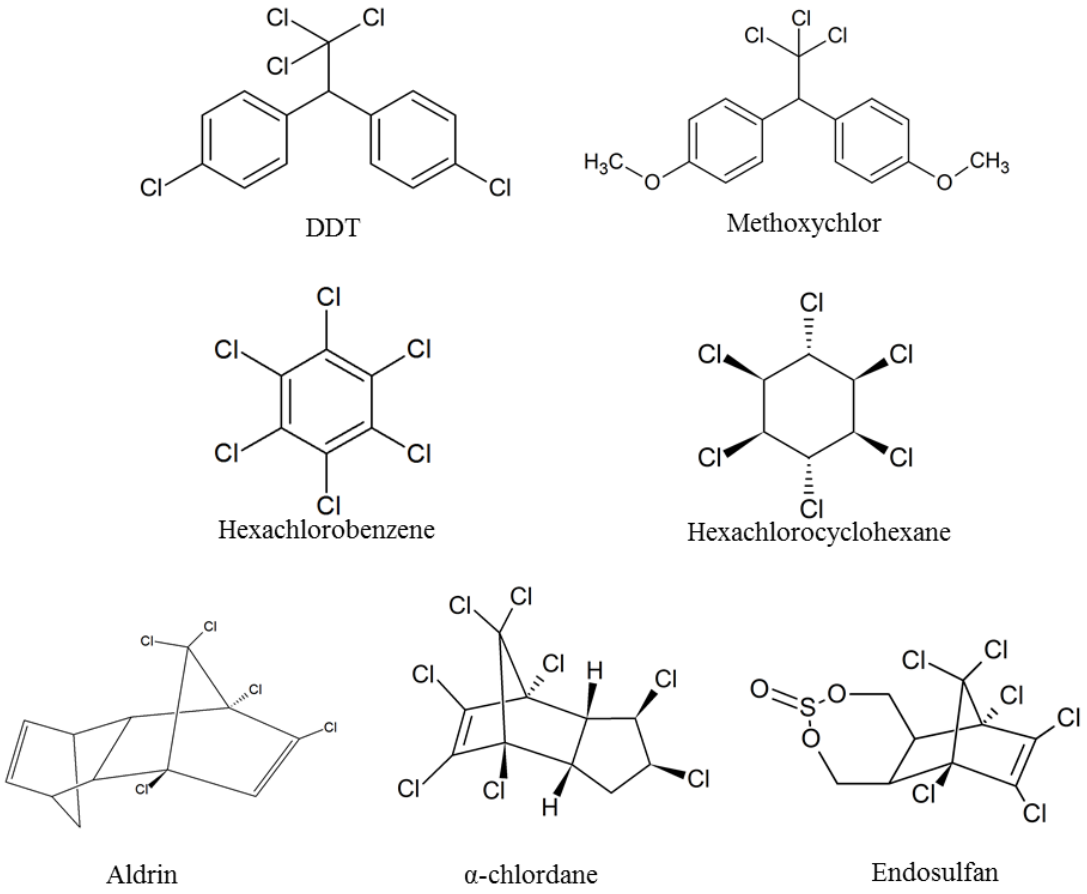
The arrows indicate rotation direction (clockwise or anticlockwise) in both benzene rings for PCBs congeners naming.

Source: Letícia A. Marques (2021).

1.1.2 OCPs – Chemical definition, terminology, and usage

Organochlorine pesticides (OCPs) are an assorted collection of organic molecules, obtained by synthetic routes, which usually contain five or more chlorine atoms in their structure, as shown in Figure 2 (MARTINS *et al.*, 2013; JAYARAJ, MEGHA, SREEDEV, 2016). They were primarily employed for agriculture purposes due to their insecticides, fungicides, herbicides, bactericides, or rodenticides properties. An estimate of FAO points that OCPs usage represents 40% of the total of all pesticides (JAYARAJ, MEGHA, SREEDEV, 2016; SUN *et al.*, 2018).

Figure 2 - Molecular structure of some OCPs compounds



Source: Letícia A. Marques (2021).

For many years, it was also common to apply OCPs in the chemical industry. Unfortunately, as chemical warfare agents, the most famous example was Agent Orange spreading during the Vietnam War. This dichlorodiphenyltrichloroethane (DDT) usage got attention to two concerning aspects – the first one, the unknown effects of OCPs on human health to that date, and the need for toxicity studies to be performed. The second one was about the urgency to regulations establishment to surveillance of production, distribution, and usage of organochlorine pesticides, or even to ban its use (BLUS, 2003; JAYARAJ, MEGHA, SREEDEV, 2016).

As mentioned, OCPs are a broad group of compounds, which could be classified as follow:

1. *Dichlorodiphenylethanes:* compounds including molecules as DDT and its derivatives, dicofol, methoxychlor, and perthane.
2. *Hexachlorocyclohexanes*: compounds as hexachlorocyclohexanes isomers and lindane.

3. *Chlorinated cyclodienes:* constituted by compounds as aldrin, dieldrin, endrin, endosulfan, heptachlor, heptachlor-epoxide, nonachlor, chlordane.
4. *Chlorinated bornanes and other molecules*: compounds such as dodecachlor, toxaphene, and chlordecone.

(SALEM, OLAJOS, 1988; MATOLCSY, 1988; ZITKO, 2003; LEÓN-SANTIESTEBAN, RODRÍGUEZ-VÁZQUEZ, 2017; RÉGO *et al.*, 2019).

The first group is the most known class of OCPs compounds, with the first production of 4,4'-dichlorodiphenyltrichloroethane (*p,p'*-DDT or just DDT) in 1943. However, a scientist named Ziegler from the Bayer company already described the production of DDT in 1873. The industrial synthesis of DDT produces various isomers as byproducts, also applied as pesticides (MATOLCSY, 1988). DDT is still used to control malaria mosquitoes in developing countries, such as Africa and India, with WHO consent (REHWAGEN, 2006; VAN DER BERG, MANUWEERA, KONRADSEN, 2017).

The class of hexachlorocyclohexanes (HCH) is constituted by compounds presenting a six-ring hydrocarbon with chlorine substituents, distributed along with axial or equatorial spots, resulting in eight HCH isomers (BLUS, 2003; LI *et al*., 2011). The chlorinated cyclodienes group has the most common characteristic of the cyclopentadiene structure, which is the first step to the chlorination process due to a Diels-Alder reaction to generate new organic polycyclic pesticides (ZITKO, 2003; MANCLÚS *et al.*, 2004). The fourth and last group includes a miscellaneous of organic molecular structures, counting with bornanes structures as toxaphene and dodecachlor, and chlordecone, which has a typical ketone structure.

Also considered persistent organic pollutants, OCPs are volatile or semi-volatile substances, quickly widespread through air mass to the environment in soil, air, water, and animal life. Bioaccumulative properties are owing to their low polarity, long-term stability, and high lipidic solubility (JAYARAJ, MEGHA, SREEDEV, 2016; LEÓN-SANTIESTEBAN, RODRÍGUEZ-VÁZQUEZ, 2017).

Whereas OCPs have several usual nomenclatures and an IUPAC name, a table of detailed OCPs terminology is presented in Table A 1 of the Appendix A - Supplementary Information, to standardize their naming in this thesis. It will be used as the first abbreviation of the referred table.

1.1.3 Toxicity of PCBs and OCPs

Many halogenated compounds have been their toxicity related to the dioxin (2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin, TCDD), the most toxic molecule of a group of planar halogenated

species. This group includes halogenated kinds of biphenyls, dibenzo-*p*-dioxins, dibenzofurans, naphthalenes, azo- and azoxybenzenes. Those compounds that promote similar toxicity to dioxin were entitled dioxin-like (DL) types. As a general rule, four lateral atoms of halogens are required to cause dioxin-like effects, and despite having been known around 419 kinds of dioxin-related compounds, only about 30 of them are considered DL species (BIRNBAUM, 1994; WHO, 2003; WHO, 2016). Most of these halogenated molecules that do not have toxicity related to dioxin are called not-dioxin-like (NDL) species.

To evaluate halogenated compounds toxicity risk, the WHO has established a scale of how much a molecule can be toxic compared to dioxin. First, it is defined the called toxic equivalent factor (TEF) for each dioxin-like compound, TEF value is evaluated based on studies of chronic toxicity performed both *in vivo* and *in vitro* and related to TCDD, which TEF value is taken as 1 for this scale settlement. Second, there is a toxic equivalent quotient (TEQ), a sum of TEFs multiplied by concentration levels of individual compounds. It means TEQ indicates the total toxicity of a sample containing dioxin and dioxin-related compounds. Regulations use TEQ values to express the maximum residue limit (MRL) for food matrices. Table 1 presents TEF values for dioxin-like compounds.

Table 1 - TEF values to chlorinated dioxin-like compounds

Compound	***WHO-TEF**
chlorinated dibenzo-p-dioxins	
2,3,7,8-TCDD	1
1,2,3,7,8-PeCDD	1
1,2,3,4,7,8-HxCDD	0.1
1,2,3,6,7,8-HxCDD	0.1
1,2,3,7,8,9-HxCDD	0.1
1,2,3,4,6,7,8-HpCDD	0.01
OCDD	0.0003
chlorinated dibenzofurans	
2,3,7,8-TCDF	0.1
1,2,3,7,8-PeCDF	0.03
2,3,4,7,8-PeCDF	0.3
1,2,3,4,7,8-HxCDF	0.1
1,2,3,6,7,8-HxCDF	0.1
1,2,3,7,8,9-HxCDF	0.1
2,3,4,6,7,8-HxCDF	0.1
1,2,3,4,6,7,8-HpCDF	0.01
1,2,3,4,7,8,9-HpCDF	0.01
OCDF	0.0003

Table 1 - TEF values to chlorinated dioxin-like compounds (continuation)

Compound	*WHO-TEF
non-ortho substituted PCBs	
PCB 77	0.0001
PCB 81	0.0003
PCB 126	0.1
PCB 169	0.03
mono-ortho substituted PCBs	
PCB 105	0.00003
PCB 114	0.00003
PCB 118	0.00003
PCB 123	0.00003
PCB 156	0.00003
PCB 157	0.00003
PCB 167	0.00003
PCB 189	0.00003

Legend: *Presented values correspond to updated WHO-TEF of 2005 (VAN DER BERG *et al*., 2006).

We can classify PCBs into three groups: Group I formed by ortho-congeners, who present estrogenic effects; Group II, constituted by mono-ortho or non-ortho substituted congeners, which have structural similarity to TCDD and also potential antiestrogenic action; and Group III holds the phenobarbital-type congeners (KLEANTHI, 2008). Also, it is possible to categorize PCBs relative to their dioxin-related toxicity. As shown in Table 1, there are twelve dioxin-like polychlorinated biphenyls (DL-PCBs) congeners with a non-ortho or mono-ortho substituted geometry.

Exposure effects to dioxin (TCDD), DL-PCBs, and other dioxin-like compounds arise mainly from a sequence of events associated with activating the aryl-hydrocarbon receptor (AhR), a ligand related to autoimmune diseases development and able to affect endocrine, reproductive, neurologic, and immune systems. There is endocrine disruption over AhR mediation, which promotes distinct inflammatory responses, especially favoring Th17 pro-inflammatory standard, seizing T-cells, or T-regulatory cells development, resulting in the negative control of the inflammatory process and promoting carcinogenic effects. Besides, PCBs can induce a series of monooxygenases dependent on cytochrome P450 through AhR or independent pathways (LANDERS & BUNCE, 1991). An ortho-substituted PCB has a coplanar conformation, which turns possible for this congener to reaches a molecular geometry similar to TCDD. So, the dioxin-like effects come from the binding ability between the coplanar congener and AhR (PUTTMANN *et al.*, 1989). In a different way of DL congeners, NDL-PCBs affect human health through other metabolic pathways, such as genic induction or direct binding

to steroidal receptor sites (NEUGEBAUER *et al*., 2015).

There is another possibility to classify PCBs according to their ability to induce specific enzymatic forms of cytochrome P450 (called isozymes) and its monooxygenases, which resulted in three classes: *(i)* 3-methylcholanthrene inducers; *(ii)* phenobarbital-type congeners; and *(iii)* mixed-type inducers, *i.e.*, molecules of PCBs whose presents properties to induce both 3-methylcholanthrene and phenobarbital routes. Coplanar non-ortho substituted congeners are mostly 3-methylcholanthrene inducers as PCBs congeners 77, 81, 126, and 169. Tri and tetra-ortho substituted congeners not-coplanar are classified as phenobarbital-type inducers; an example is PCB congener 153. An assorted of di-ortho substituted congeners present mixed-type inducer effects in animal models (using rodents and rats), *e.g.*, PCB 114 and commercial mixtures as Aroclor 1254 (ARNOLD, FEELEY, 2003; FERNÁNDEZ-GONZÁLEZ *et al.*, 2015; HENNIG *et al.*, 2001; PARKINSON, SAFE, 1987; SAFE, 2003).

It is well-known that OCPs might promote or increase the occurrence of a series of health concerns, like infertility, immune- and neurotoxicity, natural abortions, breast cancer, prostate cancer, and neurodegenerative disorders (JAYARAJ, MEGHA, SREEDEV, 2016). A mechanism of OCPs toxicity occurs over estrogen receptor (ER), in which the chlorinated compounds might act as agonists or antagonists and as antagonists on androgenic receptors (AR). More specifically hexachlorobenzene (HCB) and β-HCH also cause estrogen-like responses over AhR-dependent pathways and other autonomous routes. Furthermore, they also could operate over modulation or activation of cellular apoptosis through redox signaling changes, *i.e.*, modifications in antioxidant resistance and reactive oxygen species (ROS) accumulation eliciting oxidative stress, which in summary are a non-suitable regulation of apoptosis process, might lead to immunodeficiency, autoimmunity diseases, and cancer (MREMA *et al*., 2013).

Aldrin (ALD) is related to liver carcinomas, hepatic hyperplasia. Occupational exposure to aldrin, dieldrin (DLD), and endrin (END) increases the chances of liver and biliary cancers. DLD could inhibit the GABA (gamma-aminobutyric acid) receptor/chlorine channels and glycine channels, causing reproductive issues (JORGENSON, 2001). Other GABA antagonists are lindane (LIN) and endosulphan - they suppress the calcium ion influx, Ca-ATPase and Mg-ATPase, thus promoting the release of neurotransmitters (JAYARAJ, MEGHA, SREEDEV, 2016).

A study appointed to the fact that DDT mimics the function of estrogen hormone, inducing a cascade of enzymatic and protein production, and also has a genotoxic potential, which helps to understand the increased risk of breast cancer by this disruptive endocrine

compound (JAGA, 2000; JAYARAJ, MEGHA, SREEDEV, 2016; SINGH *et al*., 2016). Other OCPs could similarly work as endocrine disrupters since their similar four-ring steroid structure of estrogen and other hormones. There is a piece of evidence that thyroidal hormones also might be affected by OCPs and PCBs (KLEANTHI, 2008). Regardless of the controversial outcomes of many studies worldwide, a new review indicates a hypothyroid-like effect in neonates and children. Chevrier and collaborators suggest that exposure to PCBs and/or HCB could disturb thyroid function in pregnant, affecting fetal neurodevelopment (CHEVRIER *et al.*, 2008). Besides, researchers observed evidence of OCPs correlations to hyperthyroidism in men, older women, and occupationally exposed workers. At the same time, hypothyroidism was noticed in women, aging men, adolescents, and rural workers who used pesticides (LEEMANS, 2019). Another probable toxicity pathway of OCPs is hereditary epigenetics (RAITSKY, KOBLYAKOV, TURUSOV, 2000; COLLOTA, BERTAZZI, BOLLATI, 2013). Many reports associate the presence of contamination levels of following compounds β-HCH, DLD, heptachlor (HEP), LIN to a higher risk of developing Parkinson's disease, once they induce toxicity over dopaminergic neurons (FLEMING *et al.*, 1994; HATCHER, PENNEL, MILLER, 2008; SHARMA *et al.*, 2010).

1.1.4 Human exposure to PCBs and OCPs through incidents

The first case was the extensive use of pesticides as defoliation agents during in Vietnam War from 1961 to 1971. The United States has spread almost 73 mi liters of herbicides. Orange Agent is the most famous mixture between the "rainbow herbicides", being composed of one part of 2,4-D (2,4-dichlorophenoxy)acetic acid) and one part of 2,4,5-T (2,4,5-trichlorophenoxy)acetic acid) and corresponding to 62.63% of the total herbicide amount sprayed (STELLMAN *et al.*, 2003). Pulverization was performed using spray trucks, helicopters, or even hand sprayers by the soldiers. It is estimated that 4.8 million people in Vietnam were exposed to some of the rainbow herbicides, and from this amount, over 3 million still suffering diseases to Agent Orange or dioxin. It is also important to remind that US soldiers had suffered exposure to these compounds; however, there is no sufficient information about the location or degree of exposure. Even conducted studies with American veterans present dubious conclusions since they contemplated a small size population. This lack of information affects statistical correlations, so it impossible to state how many US veterans were affected (SMALL, 2019).

Several disorders experienced along these years by veterans were recognized as associated with Agent Orange and its related mixtures by the five countries of Allied Forces

such as chloracne, diabetes type 2, ischemic heart disease, lymphoma, sarcoma, and many other types of cancers (YOUNG, YOUNG, 2017).

In 1968, an incident occurred in the cities of Fukuoka and Nagasaki, western Japan. An unknown disease was first reported with symptoms as chloracne, skin pigmentation, and eye discharge. PCBs contamination of a local rice oil brand for cooking use and sold as feed supplement for poultry was discovered. That disease was named Yusho, which means "oil disease" in Japanese. Commercial PCB oil had been used for coolant purposes in the factory and somehow leaked out, contaminating the cooking oil. More than 14,000 people were affected by PCB poisoning, with 2000 severely ill victims. Also, the massive death of the poultry population was noticed. Implementation of an annual health monitoring of Yusho patients was established after the episode. Increased fetal death rate and a higher incidence of lung and liver cancer on males were observed. Blood levels of dioxin-like compounds decreased over the years (ONOZUKA *et al.*, 2009; YOSHIMURA, 2012). Even half-century later of the first episodes of Yusho disease, scientists still investigate Yusho patients' second generation to understand dioxin-like compound effects through generations (MITOMA *et al.*, 2015). In any case, Yusho disease was an opportune episode to get the scientific community concerned about the real need investigation of PCBs and dioxin-like compounds toxicity, their effects on human health, and the necessity of legal regulations establishment to monitor them.

Unfortunately, a similar situation happened in Taiwan just a couple of years later of the Yusho disease event. In 1979, rice oil contamination took place on the same industrial process failure as occurred before in Japan, and it becomes known as Yusheng disease. About 2000 people were contaminated who have presented similar symptoms to Yusho disease (LI *et al.*, 2013).

In 2004, a candidate for Ukraine's presidency, Victor Yushchenko, was poisoned with TCDD during a campaigning dinner in Kyiv. Just a few hours after the dinner, he felt acute stomach pains, headache, and vomitus. He also faced large pancreatitis, hepatitis, gastrointestinal inflammation in the early 12 months of the poisoning. During the three first weeks of poisoning, he received a non-specific treatment since the poisoning agent was unknown. Finally, the appearance of chloracne helped to elucidate the poisoning agent (SAURAT *et al.*, 2012; YOUNG, REGENS, 2005). Hereafter, it was possible to improve medical care services to increase Yuschenko's life quality. In the first months, he had presented a 108000 pg of TCCD per gram of lipid in his serum, equivalent to 5-million times than the acceptable daily ingestion. In the next three years, samples of fat, serum, feces, and skin samples

were collected and monitored not just to TCDD levels but also their hydroxylated metabolites (SORG *et al.*, 2009).

1.1.5 Regulations

It is undeniable that the launch of the book “Silent Spring” by Rachel Carson in 1962 got attention from countries authorities for the non-regulated use of organochlorine substances in agriculture and industries (JEPSON, LAW, 2016). Also, the 1970's episodes of human exposure to PCBs and OCPs were an impellent to establishing regulation laws.

The first significant conquest against organochlorine use was accomplished in July 1972 when DDT was banished in United States (US) after years of extensive use as a biological weapon in the Vietnam War. A few years later, on the other side of the Atlantic Ocean, a legal act took place in 1976 at European Commission (EC) by Council Directive 76/769/EEC, that recognized the potential of PCBs on damaging human health and highlighted the need for its limited usage, production, and transportation. Nowadays, the Directive 76/769 is no longer in force, updated by the 2008 latest version.

Commission Directive 2006/77/EC was another important mark for environmental and health politics, once it rules about maximum levels of OCPs compounds in animal feed, limiting its use (EUROPEAN COMMISSION, 2006). Back in 1979, the US government implemented the Toxic Substances Control Act (TSCA), banning PCBs manufacture, industrial processing, usage, and distribution in their territory. A couple of years later, Brazil ruled their first restriction over PCBs usage, and it is Interministerial Ordinance No. 19 from January 2nd, 1981; after this, it has been sanctioned Federal Law no. 7802 from 1989, which provisions about the regulation of pesticides in general and posteriorly by Acts 4074/2002 and 5981/2006 (JARDIM, CALDAS, 2012). In Asia, as an enormous economy based on agriculture, China banned DDT, HCH, ALD, DLD, and HEP application as pesticides only in 1983, but not after producing 20% of worldwide total DDT. However, chlordane (CLD) usage remained legal until 1999 (TIEYU *et al.*, 2005; WONG *et al.*, 2005; SUN *et al.*, 2018).

In 2001, the United Nations Environmental Program (UNEP) organized the Stockholm Convention, which reiterated twelve compounds (named "The Dirty Dozen") and its groups as a priority of monitoring and classified as POPs. Four features were considered to classify a POP, being their great persistence in the environment, high dissipation through air and water, lipophilic bioaccumulation, high toxicity, even at low concentrations. Due the capability of dioxin-related compounds as PCBs and the organochlorine pesticides to check every box on the Stockholm POP features, they did belong to the original molecules dealt in the Convention, or

it had been added in 2009 and 2012 (JORGENSON, 2001; TANG, 2013). Endosulfan and its isomers were the most recent banned compounds between organochlorine pesticides class, which happened in mid-2012 after the Stockholm Convention; however, it was allowed exemptions to its use on 22 crops for the next five years – extending this situation up to 2017 (HOGUE, 2011).

The present law of the Commission Regulation from EC no 1881 of December 19th, 2006, establishes the MRL for various food matrices, as 6.0 pg/g of fat for PCBs in milk. In the United States, currently, Food and Drug Administration (FDA), in a joint effort with the Environmental Protection Agency (EPA), enforce laws about limits of organochlorines residues for human food or animal feed. The specific values of contamination in different food matrices are listed in the 40 Code of Federal Regulations (CFR) Part 180, named as "Tolerances and Exemptions for Pesticide Chemical Residues in Food" (U.S. e-CFR, 2021).

In Latin America, developing countries have been following Brazilian legislation over pesticides and PCBs for food matrices and health risk appraisal. Currently, we have two strategies to monitor organochlorine compounds in Brazil, known as the Program for Pesticide Residues Analysis in Food (PARA, *from Portuguese "Programa de Análise de Resíduos de Agrotóxicos"*), directed by the Brazilian Health Regulatory Agency (ANVISA, from Portuguese "*Agência Brasileira de Vigilância Sanitária"*), an autarchy linked to the Ministry of Health, and the National Plan for Control of Residues and Contaminant (PNCRC, from Portuguese *"Plano Nacional de Controle de Resíduos e Contaminantes"*) from the Ministry of Agriculture, Livestock, and Supply (MAPA, from Portuguese *"Ministério da Agricultura, Pecuária e Abastecimento"*) (JARDIM, CALDAS, 2012; BRASIL, 2020).

Handford and coworkers (2015) review appoints difficulties in harmonizing pesticide legislation for foodstuff worldwide, which creates commercial trade problems (HANDFORD, ELLIOT, CAMPBELL, 2015). Many developing nations hold on agricultural exportation as the main economic activity, so pesticide use is still a tool for increasing rural production. Additionally, there are not enough laboratories and facilities to check out pesticide usage restrictions; therefore, developing nations struggle to the attendee with rigid stringent regulations enforced by EC and the US (ECOBICHO, 2001).

1.1.6 Latest twenty years of PCBs and OCPs contamination in milk

Despite the prohibition of PCBs and OCPs usage since the 1970s, they are still found on the environment, so the international scientific community continues to perform research monitoring these chlorinated compounds in human breast milk. From 1951 to 1999 more than

130 works of DDT quantification in human milk were performed worldwide, and Smith (1999) created a compilation table of all of them. It was possible to notice that DDT levels in breast milk have been significantly decreased over the years in all countries, assigned to DDT usage restriction (SMITH, 1999). Pirsaheb and coworkers (2014) also published a comprehensive revision of OCPs presence in breast milk between 1980 and 2013 (PIRSAHEB *et al.*, 2015). In its turn, Olisah and colaborators performed a comprehensive review of OCPs in biological and environmental matrices from 1992 to 2018 (OLISAH, OKOH, OKOH, 2019).

A study conducted in 2007 in two Zheijang provinces from China presented alarming PCBs and OCPs contamination in human milk, especially PCB 138, p,p'-DDE, and DDT (ZHAO *et al.*, 2007). Tunisian women from rural or urban areas presented PCBs, p,p'-DDE, DDT, HCH, and HCB levels on breast milk to exceed tolerable WHO daily intakes (ENNACEUR, GANDOURA, DRISS, 2008). The previously cited review of Pirsaheb appointed to the presence of two or more OCPs in breast milk samples majority worldwide (PIRSAHEB *et al.*, 2015). In Egypt, a buffalo milk study showed that methoxychlor (MTX) and HCB are higher than MRL, while DLD concentration was below its values (SHAKER, ELSHARKAWY, 2015). In 2017, research conducted in Tanzania showed breast milk contamination with PCBs and OCPs higher than acceptable levels. For instance, dieldrin was detected up to 937 ng g^{-1} of lipid weight (lw), while PCBs presented up to 157 ng g^{-1} lw. (MÜLLER *et al.*, 2017).

On the other hand, there are many papers in the literature about the decline of these chlorinated compounds levels in milk matrices. Part of these articles is the discussion of PCBs and OCPs concentrations exceeding MRL or toxic equivalent quotient (TEQ from WHO), which seems controversial to diminishing trends in the latest decades.

In a review paper of Chinese DDT usage, the authors highlight decreasing 27% of p,p'-DDE+DDT levels between 1976 to 1985 and 80% from 1985 to 1999 (WONG *et al.*, 2005).

In Taiwan, a study of 2006 reported decreasing DDT concentrations in human milk amongst the previous two decades. For example, the sum of DDT concentrations decreased from 3595 to 333 ng g^{-1} lw in early two decades of this publication (CHAO *et al.*, 2006). An Australian research detected the following mean levels of OCPs in human milk samples: 311 ng g^{-1} lw of p,p'-DDE, 16 ng g^{-1} lw of DLD, 18 ng g^{-1} lw of HCB, and 9 ng g^{-1} lw of DDT. The contamination was observed despite a tendency of decline OCPs levels since the '90s observed by this research' authors (MUELLER *et al.*, 2008). Breast milk samples of *primiparae* mothers from Israel were evaluated in a pool of samples, and this study did not find 16 pesticides at detectable levels, including ALD, END, and dodecachlor. Authors also compared

their POP levels results for pooled samples with data from other four countries (Ireland, Belgium, Germany, Tunisia, and the USA), obtaining the lowest values for: DDT (4.3 ng g^{-1} lw); DLD (2.8 ng g^{-1} lw); PCBs congeners 118, 138, 153, 180 (4.1, 6.0, 9.8, 4.8 ng g^{-1} lw, respectively); and also for the sum of PCBs indicators (23.9 ng g^{-1} lw) (WASSER *et al.*, 2015). Lu and collaborators found significant DDT levels and metabolites, HCB, and HCH in human breast milk. The concentrations of HEP, CLD, ALD, END, DLD were extremely low. They also indicate the diminishing of OCPs levels compared with 2002 and 2007 (LU *et al.*, 2015). A research carried out in 2016 in Saudi Arabia noticed organochlorinated compounds HEP, DLD and END presented levels in human milk enough to exceed recommended acceptable daily intake values (set as 0.1 µg kg^{-1} body weight day^{-1}) (HAJJAR, AL-SALAM, 2016).

A WHO global monitoring report of 52 countries indicates that recent human exposure to DDT levels through human milk intake is tolerable. On the other side, concentrations of dioxin-related compounds, including DL-PCBs, are higher than secure TEQ values (~ 0.1 pg g^{-1} lipid) established for the fetus breastfed infants (VAN DEN BERG *et al*., 2017).

In addition, several recent studies show electronic waste (e-waste) recycling sites as the new hot spots of environmental contamination (ROBINSON, 2009; FU *et al*., 2011; ZHANG, WU, SIMONNOT, 2012). As consequence formal and informal workers suffer from occupational exposure to these halogenated compounds at the workplace. Considerable levels of PCBs had been found on human breast milk from women workers, with PCBs levels ranging from 28 to 59 ng g^{-1} lw in Vietnamese mothers (workers from Bui Dau e-waste site), and from non-detected to 57.6 ng g^{-1} lw in the breast milk of Chinese women (workers from Guiyu e-waste) (TUE *et al*., 2010; XING *et al*., 2009). In Ghana's capital, Accra, a large e-waste dumping site and a recent study reported contamination of PCBs and polybrominated compounds on cow milk from cattle of that region (ASANTE *et al*., 2010). Also, Asamoah and collaborators found higher PCBs levels in breast milk samples from women workers of Agbogbloshie e-waste than in those samples from women of a residential area, respectively 3.64 and 0.03 ng g^{-1} lw (ASAMOAH *et al*., 2018).

1.2 Extraction techniques for PCBs and OCPs from milk

Milk is a complex matrix due to its composition, heterogeneity (dependent on the product processing status), and cattle genetic variability. Its nutritional composition contains lipids, which constitutes around 3 to 4% of milk solids content, including fatty acids, phospholipids, glycosphingolipids, proteins, which are about 3.5% of milk solids with caseine representing 80% of this amount, carbohydrates, such as lactose; vitamins and antioxidants, as

vitamins A, E, B12; and minerals as calcium, selenium, magnesium (HAUG, HØSTMARK, HARSTAD, 2007; GRĂDINARU, CREANGĂ, SOLCAN, 2015; FAO, 2020). Unfortunately, it is common to found high concentrations of hormones, antibiotic, and organochlorine residues in animal milk.

Human breast milk is different from other mammals' milk both in composition and concentration of ingredients. For instance, human milk presents a lower nutritional value than other mammalians milk; however, it holds a higher carbohydrate content. Lactose levels might reach 6.4 to 7.6 g per 100 mL in the mature milk (JENNESS, 1979; PRENTICE, 1996). The most present proteins are caseine, α-lactoalbumin, serum albumin, and immunoglobulin A (IgA antibody). Fat composition is between 3 to 5% in human breast milk, superior to animal milk, and the majority of its triglycerides consisting of palmitic (16:0) and oleic (18:1) acids (BALLARD, MORROW, 2013; ANDREAS, KAMPMANN, LE-DOARE, 2015). The fat content of human breast milk might also bioaccumulate contaminants, particularly lipophilic compounds such as chlorinated ones.

Nevertheless, the fatty composition of milk samples presents a challenge – it will be required a sample preparation step to promote a better identification and greater quantification of the analytes. In addition, the sample prep step performs analytes pre-concentration and/or removes interferences compounds from the matrix, improving signal-noise quality and preserving the equipment life cycle (DIMPE, NOMNGONGO, 2016). Unfortunately, in commercial milk, despite international regulatory agencies establishing MRL values for a range of contaminants, there is no standardization about sample preparation techniques that must be used. Thus, in the next sections, we will discuss the efforts from the scientific community to develop sample preparation techniques for PCBs and OCPs determination in both animal and human breast milk.

1.2.1 Classic extraction methods

There are many procedures to perform sample preparation of chlorinated compounds that show easy execution, low cost, and simple instrumentation. For many years, liquid-liquid extraction, solid-liquid extraction, or Soxhlet extraction were the most applied for researchers aiming OCPs and PCBs removal from the targeted matrices.

According to Martins *et al*. (2013), the most widespread method for OCPs extraction is liquid-liquid extraction (LLE) (BUSZEWSKI, SZULTKA, 2012; MARTINS *et al*., 2013). The principle of LLE is partition equilibrium of an analyte between two immiscible solvents. A physicochemical description of this extraction process uses distribution coefficient, K_D, given

by the ratio of analyte concentration C_A in a solvent A and analyte concentration C_B in a solvent B (GOLUMBIC, 1951), as given by Equation 1:

$$K_D = \frac{C_A}{C_B} \qquad \textit{Equation 1}$$

To execute a LLE batch, a simple apparatus as a separating funnel is needed. Sample must be soluble in solvent A, placed in the separating funnel, and then a solvent B (immiscible or partially miscible to the first solvent) is added. The system must be vigorously shaken, left to repose until a noticeable formation of two phases, and then mechanically separated through the drain valve. We highlight the importance of a careful choice of solvents to obtain as higher K_D as possible. Continuous LLE is a multi-step LLE batch, which means that the LLE process must be repeated for many hours. Therefore, a large apparatus is necessary for continuous LLE, containing an extractor, a condenser, and a collector recipient (GOLUMBIC, 1951).

Some experimental factors play essential roles in a successful LLE procedure, such as: avoid emulsion formation or eliminate it through salt addition, surfactants, or even using filtration/soft stirring; adding of dryness agents or inorganic salts; an occurrence of salting-in or salting-out effect, *etc*. In addition, it is required basic laboratory facilities and low-cost instrumentation to execute LLE, sparing an expensive labor cost of a well-trained analyst once it is easy to execute. However, LLE limitations include the usage of large solvent volumes, time-wasting process, high exposure of the analyst to the solvents, and it is not environmental-friendly or a Green Analytical technique (BEYER, BIZIUK, 2008; RAWA-ADKONIS, WOLSKA, NAMIEŚNIK, 2006). Moreover, LLE is not feasible for automatization, coupling to separation techniques is difficult to achieve, and it presents a low selectivity, triggering the decline of its applications nowadays.

An investigation of 27 breast milk samples from polish *primiparae* or *secundiparae* mothers for PCBs and OCPs were carried out with a liquid-liquid extraction with the milk fat content determination by gravimetry, and gas chromatography (GC) - electron capture detection (ECD) analysis. PCBs contamination levels on polish *primiparae* mothers were slightly higher than in the *secundiparae* milk samples. PCB 153 was the most dominant congener, while p,p'-DDE was the most abundant between the OCPs species monitored (SZYRWIŃSKA, LULEK, 2007).

Solid-liquid extraction (SLE, or supported solid-liquid extraction, SSLE) is also a technique dependent on the distribution ratio, similarly to the LLE. A partition of the analyte from the aqueous samples between the solid inert support and the solvent will flow through the supported matrix to extract it in SLE methods. To perform SLE with liquid milk, it is required

to mix the sample with anhydrous sodium sulfate to generate a coarse powder, and then carry on to the typical SLE procedure. As a classic method, solid-liquid extraction is also labor-intensive, expensive, and not environmentally friendly considering large solvent volumes used (CHUNG, CHEN, 2011; MARTINS *et al*., 2013; DANACEUA, HAYNES, CHAMBERS, 2017).

An extensive study was carried out to monitor OCPs and PCBs in breast milk from German women for twelve years by Schade and Heinzow (1998). A total of more than 3500 samples were collected and analyzed by GC-ECD after performing SLE extraction using Florisil™ (an activated magnesium silicate) as a sorbent phase and an elution mixture of 2-methyl pentane and dichloromethane (80:20, v/v). The researchers noticed a significant decreasing trend in levels of chlorinated contaminants through these years – a reduction of 81% to DDT levels, a decrease around 80% for HCB and β-HCH average concentrations, and a diminishing of 60% in PCBs values (SCHADE, HEINZOW, 1998). This tendency is attributed to the efficacious chlorinated compounds ban measures in the '70s on the European Community, also observed in Sweden, Norway, and Spain. A questionnaire was also applied to the sample donators to assemble personal data as age, parity (*primi*-, second-, or *multiparae* women), weight, height, duration of previous breastfeeding experiences, etc. Since former breastfeeding might be seen as an important excretion route of these persistent residues, lower concentrations of organochlorine compounds are expected to follow breastfeeding from one woman (SCHADE, HEINZOW, 1998).

A similar long-term surveillance research about OCPs and PCBs monitoring in breast milk from Japanese mothers was conducted from 1972 to 1998. First, a Florisil column was used to extract milk fat, and then OCPs were extracted using *n*-hexane. A concentrated aliquot (3 to 5 mL) of the eluate was mixed with 15g of Florisil, followed by elution using *n*-hexane/diethyl ether (9:1, v/v). GC coupled to mass spectrometry (GC-MS) was carried out. Reduction of OCPs and PCBs levels is a general direction through the years; however, an unregular diminishing trend to chlordane is observed once its former use as termite insecticide remains at Japanese houses (KONISHI, KUWABARA, HORI, 2001).

1.2.2 Solid Phase Extraction

Established about 50 years ago, solid-phase extraction (SPE) is a sample preparation technique based on liquid-solid separation. The traditional SPE extraction device is a polypropylene cartridge, similar to a syringe containing a sorbent phase packed inside. Manual or automatized pressure systems pass-by samples through the stationary phase of the cartridges,

and then a solvent is percolated over the sorbent phase bed to obtain a fraction enriched of analyte (BEYER, BIZIUK, 2008). A vacuum system is commonly used to assemble multiple SPE cartridges and perform a simultaneous sample preparation. About extraction process, we can describe its occurrence in four steps: *(i)* conditioning; *(ii)* sample application and analyte retention; *(iii)* clean up, for interferent compounds remotion; and *(iv)* elution of analytes using small solvent volumes (BUSZEWSKI, SZULTKA, 2012; ANDRADE-EIROA *et al*., 2016).

Different SPE formats are commercially available, like cartridges, disks, 96 well-plates, pipette tips, *etc*.; all of them are single-use devices, and disposables. SPE separation mechanisms are the same as classic liquid chromatography (LC), like adsorption, partition, ion exchange, and size exclusion (POOLE, 2003). In adsorption (or normal phase), the analyte establishes an adsorption equilibrium to the sorbent surface, staying retained until elution. The most common stationary phases are silica, alumina, diatomaceous earth, and Florisil™. Partition, also called reversed phase (RP), is the most used separation mechanism and consists of the analyte equilibrium with the interior from stationary phase. Cartridges of SPE based on partition are produced with chemically bonded phase as support particles (in general, silica) coated with functional groups as octadecylsilane (C18 or ODS), octylsilane (C8), *etc*. Water, saline buffers, organic solvents, or mixtures of them are typically utilized to perform the elution (THURMAN, MILLS, 1998). Ion exchange has a retention process grounded at the reversible electrostatic attraction between sample ions and opposite charge cores immobilized in the stationary phase. In this case, elution solvents must have counter ions to be exchanged with the counter ions core; besides, it must present stronger attraction for the ionic exchange resin than for the analyte, so the analyte can be eluted. Size exclusion is the only separation mechanism that depends on the physical interaction between analytes and the pore size from the sorbent phase.

SPE presents several advantages, as can be cited: *(i)* the possibility of simultaneous extraction of many samples; *(ii)* low volumes of solvent compared to LLE or SLE; *(iii)* does not occur emulsion formation; *(iv)* high selectivity due to diversity of SPE phases. Besides, it also offers great recovery, and easiness automation. On the other hand, we can assign as limitations of SPE the large solvent volumes used; time-consuming process; low reproducibility; high exposure of the analyst; the high cost of each disposable SPE device; the expensive automatization of SPE procedure; and potential elution flow blockages (RAWA-ADKONIS, WOLSKA, NAMIEŚNIK, 2006). Thus, it becomes clear the necessity for sample preparation miniaturization. Furthermore, experimental factors might affect SPE performance, especially selecting the elution solvent and sorbent phase (toward particle size and polarity).

Sample volumes to be applied and compatibility between SPE device format and analyte concentration in the matrix are important aspects to be considered during the development of an SPE-based method.

Jaraczewska and collaborators (2006) had evaluated PCBs and OCPs in human samples including breast milk from Polish mothers. First, a gravimetric procedure to remove the milk fat content was carried out, followed by SPE extraction taking on Oasis™ SPE cartridges and dichlorometane to eluate; after detecting the chlorinated contaminants, GC-MS analysis with electron capture negative ionization (ECNI) was performed. The obtained sum of all fifteen PCBs congeners was 153 ng g^{-1} lw, with congeners 153, 180, and 138 having the highest contribution to these values. Even higher was the sum of OCPs levels; to cite, alone p,p'-DDE presented a mean concentration of 817 ng g^{-1} lw. Likewise, milk showed to be the most contaminated matrix of this study compared to the two other samples also assessed – maternal serum and human umbilical cord serum (JARACZEWSKA *et al*., 2006).

For OCPs investigation in milk and milk powder, Zheng *et al*. (2014) used an arrangement of gel permeation chromatography (GPC) to SPE to decrease the presence of lipidic interferents. Florisil™ cartridges of 1000 mg were used, and elution had been performed by the mixture of 5% (v/v) of *n*-hexane in dichloromethane. Sample preparation procedures were optimized, and the GC-MS/MS was used as method of quantification; after validation, researchers analyzed 50 samples of milk and milk powder from the local market; however, none of the analytes were even detected (ZHENG *et al*., 2014).

A recent study reports a tandem approach to perform SPE extraction of dioxin-like compounds from human milk, including eighteen PCBs congeners. The researchers hyphenated two lab-made SPE cartridges – the first one was an acid silica SPE. The second was an activated basic silica SPE; isotopic-labeled analysis was carried out in a GC-MS, using different ionization energy than the usual (42 eV). Proper validation parameters were obtained for the tandem-SPE, and then ten human breast milk samples were evaluated using it. All samples present NDL-PCBs levels higher than the limit of detection (LOD) values, which varied from 0.04 to 0.07 ng g^{-1} lw. The authors also established a comparison between tandem-SPE and an automated sample cleanup system (ASCS). Excellent correspondence was verified among them, with the advantage of tandem-SPE being a faster sample prep step and less solvent-consuming (LIN *et al.*, 2016).

Huang e coworkers (2017) also employed a GPC-SPE procedure to extract PCBs, OCPs, and other persistent organic pollutants using deactivated SiO_2 SPE cartridges, elution solvent being *n*-hexane (first fraction) or a mixture of one part of *n*-hexane and one part of

dichloromethane (second fraction), with posterior GC with sequential mass spectrometry (MS/MS) analysis. After monitoring 20 human milk samples, DDT and related compounds were the most abundant followed by HCH isomers, presenting average concentrations levels of 140 and 503 ng g^{-1} lipid, respectively. PCB congeners 28 and 153 were the most predominant in their class (HUANG *et al*., 2017).

1.2.3 Solid Phase Micro Extraction

Solid-phase micro extraction (SPME) is a non-exhaustive sample preparation technique developed by Janusz Pawliszyn and collaborators in the early '90s (ZHANG, YANG, PAWLISZYN, 1994). A needle-sized wire covered with a polymeric phase promotes the analyte extraction from the sample matrix. Originally it was used a fused silica capillary and the device named SPME fiber. Nowadays, SPME has many device shapes, as disk, membranes, tube (known as *in-tube* SPME), even with suspended particles. SPME film type and thickness are essential factors in the sample prep optimization process once it affects extraction selectivity. Other aspects as sample volume, addition of co-solvents, ionic strength of the matrix, pH of extraction, equilibrium time, and temperature of extraction are also significant to the performance of the SPME process (VALENTE, AUGUSTO, 2000; PAWLISZYN, 2000; ABDULRA'UF, TAN, 2015). Some advantages of SPME are easy operation, good selectivity, high sensibility, absence of solvent usage, low cost, and simple automation or coupling to separation techniques (PAWLISZYN, 1997).

Extraction in SPME is based on the equilibrium between analytes and the polymeric phase film covering the device, so it is possible to describe the process thermodynamically (PAWSLIZYN, 2012). Then, the initial concentration of the analyte in the sample, C_0, can be defined by:

$$C_0 V_S = C_S^{\infty} V_S + C_f^{\infty} V_f \qquad \text{Equation 2}$$

Where C_0 is the initial concentration of the analyte in the sample; C_f^{∞} is the concentration of the analyte in the polymeric coating; C_S^{∞} is the concentration of the analyte in the sample on equilibrium, V_s is the volume of the sample, and V_f is the volume of the fiber polymeric coating.

In the equilibrium, this system will be described by the distribution coefficient of analyte between polymeric phase and sample matrix, K_{fs}, given by Equation 3:

$$K_{fs} = \frac{C_f^{\infty}}{C_S^{\infty}} \qquad \textit{Equation 3}$$

Rearranging Equations 2 and 3, we have Equation 4:

$$C_f^{\infty} = C_0 \frac{K_{fs} V_S}{K_{fs} V_f + V_S} \qquad \textit{Equation 4}$$

$$n = C_f^{\infty} V_f = C_0 \frac{K_{fs} V_S V_f}{K_{fs} V_f + V_s} \quad \textit{Equation 5}$$

Usually, the volume of the sample is much bigger than polymeric phase volume, therefore:

$$V_s \gg K_{fs} V_f \quad \textit{Equation 6}$$

$$\therefore \quad n = C_0 K_{fs} V_f \quad \textit{Equation 7}$$

Thereby, we can notice that analyte amount of substance is independent of sample volume.

Compounds of high or medium volatility can be extracted performing the headspace (HS) mode of SPME. In this approach, analytes are contained in the gas phase, which is in equilibrium with the condensed phase. Thus, it is easy to combine to GC-ECD or GC followed by mass spectrometry or sequential MS/MS for trace levels analysis as PCBs and OCPs in foodstuff and environmental samples (ZHANG, PAWLISZYN, 1996; MARTINS *et al*., 2013).

Considering a system constituted of polymeric coating of SPME fiber, headspace, and matrix sample, analytes migration will be the phenomenon between these three phases until equilibrium was reached. When it happens, we could express the whole system as in Equation 8:

$$C_0 V_S = C_S^{\infty} V_S + C_h^{\infty} V_h + C_f^{\infty} V_f \quad \textit{Equation 8}$$

Where C_h^{∞} e V_h were, respectively, analyte concentration at equilibrium in headspace phase and headspace volume.

Since K_{fh} is the distribution coefficient of analyte between polymeric film (fiber coating) and headspace, and K_{hs} the distribution coefficient of analyte between headspace and sample matrix, described by Equations 9 and 10, respectively:

$$K_{fh} = \frac{C_h^{\infty}}{C_f^{\infty}} \quad \textit{Equation 9}$$

$$K_{hs} = \frac{C_h^{\infty}}{C_S^{\infty}} \quad \textit{Equation 10}$$

At once, permutating Equations 9 and 10 to Equation 8, the mass of analyte present in the polymeric coating can be described by:

$$n = \frac{K_{fh} K_{hs} V_f C_0 V_S}{K_{fh} K_{hs} V_f + K_{hs} V_h + V_s} \quad \textit{Equation 11}$$

and,

$$K_{fs} = K_{fh} K_{hs} = K_{fg} K_{gs} \quad \textit{Equation 12}$$

Where K_{fg} is the distribution constant between fiber coating and gaseous headspace, and K_{gs} is the distribution constant of the gaseous headspace and the sample. Equation 12 is stated since K_{fs} can be approximated by the K_{fg}, and K_{hs} by the K_{gs} since moisture in the headspace can be disregarded (PAWSLIZYN, 2012).

Finally, the resulting expression is given by:

$$n = \frac{K_{fs}V_f C_0 V_S}{K_{fs}V_f + K_{hs}V_h + V_s} \qquad \textit{Equation 13}$$

Following this mathematical description of HS-SPME, it is possible to summarize a few applications of this extraction technique to organochlorines molecules analysis from milk samples.

A saponification HS-SPME method with analysis by GC-ECD and GC-MS/MS was proposed for 9 PCBs congeners from bovine milk. The strategy of adding NaOH to promote saponification of fat components improved extraction between 3.0 to 8.2 times compared to the absence of the saponification approach. The method was tested with liquid milk samples spiked of the certified material reference (full-fat powdered milk), showing recovery rates concordant to reference values (LLOMPART *et al*., 2001).

González-Rodríguez and collaborators (2005) proposed an SPME method to determine 40 pesticides, including DDT and metabolites, MTX, DLD, in distinct milk samples. The authors performed a comparison between direct immersion and headspace modes to SPME execution with low-pressure GC-MS/MS determination. They successfully applied the proposed method to analyze 15 commercial cow milk, 17 goat milk, and three breast milk samples. Some of these samples were contaminated with p,p'-DDE and DDT levels; however, concentrations were lower than LOQs (GONZÁLEZ-RODRÍGUES *et al*., 2005).

Bovine milk was evaluated using HS-SPME for 35 pesticides, including the OCPs *cis*-CLD, *trans*-CLD, MTX, END, ALD, α-HCH, β-HCH, LIN, p,p'-DDD, p,p'-DDE, DDT, DLD, HEP, HPX. A 2^5 experimental design was used to optimize extraction conditions, and analysis was carried out in a GC-μECD, followed by successful method validation. However, real samples from dairy cattle of the northwest of Spain did not present pesticide residues levels, except for one sample that showed 1 ng mL^{-1} of chlordane (FERNANDEZ-ALVAREZ *et al*., 2008).

Joshi *et al*. (2014) studied the presence of 21 PCBs congeners in milk and water samples using polymeric ionic liquid (PIL) sorbents to cover SPME fibers with GC-ECD-MS/MS analysis. The proposed PIL favors π–π interactions between PCBs molecular structure and the fiber coating since aromatic moieties are added to PIL synthesis. So increased sensitivity and

sensibility were observed to extract PCBs from milk using PIL-fibers compared to commercial PDMS-fiber (JOSHI *et al*., 2014).

Kowalski and co-authors investigated human breast milk from twenty Brazilian *primiparae* and *multiparae* mothers for twelve PCBs presence using HS-SPME with GC-ECD analysis. The following SPME conditions were used: commercial 100 µm PDMS fiber; 5 mL of milk sample; 5.0% of methanol as co-solvent; sodium chloride (36% m/v) to increase ionic strength; 10 min HS equilibration time; extraction period between 40 to 100 min; desorption for 5 min at 280ºC in the GC injector. They also evaluated temperature ranged from 45 to 95 °C for sample extraction. One study was conducted using experimental design to optimize the PCB extraction (KOWALSKI *et al*., 2007, 2010). The second used an artificial neural network (ANN) for data treatment (KOWALSKI *et al*., 2013). They found PCBs levels (sum of 12 congeners) varying from 0.6 to 13.9 µg L^{-1} in the breast milk samples from Brazilian women living in industrialized areas, but not in those from the mothers of the Amazon region (KOWALSKI *et al*., 2010).

1.2.4 QuEChERS

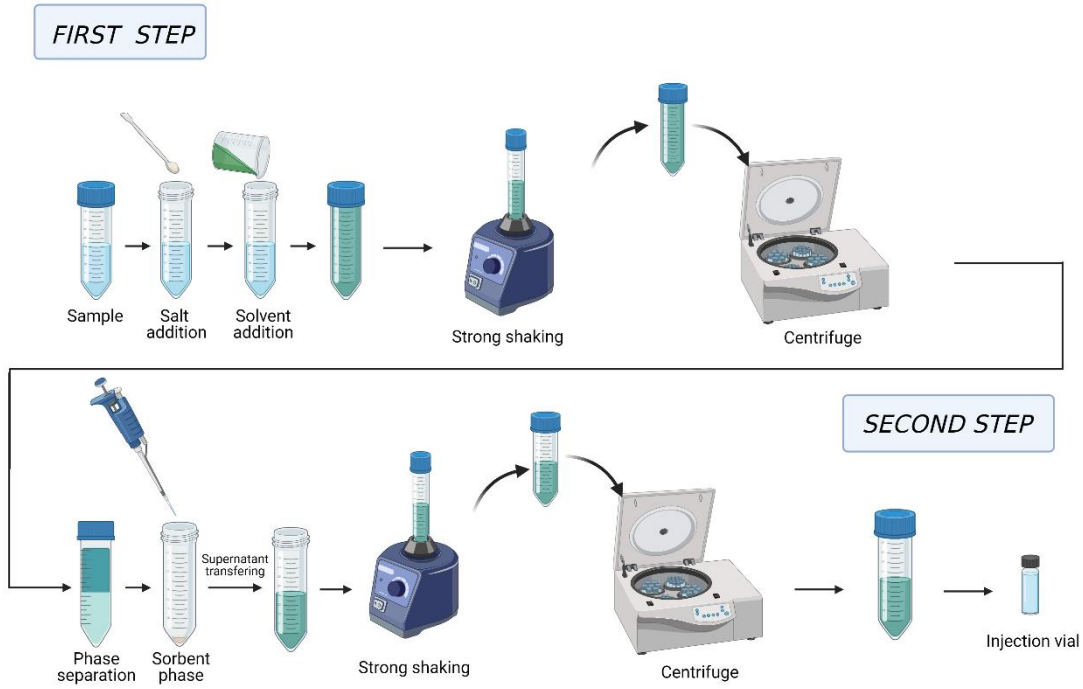
Originally conceived in the early 2000s, the QuEChERS procedure has been planned to perform multiclass or multi-residue pesticides analysis in foodstuff with low cost and good analytical frequency. QuEChERS is an exhaustive sample preparation method based on partition extraction of analytes in a liquid phase, followed by a dispersive solid-phase extraction (d-SPE). The acronym QuEChERs means Quick, Easy, Cheap, Effective, Rugged, and Safe (ANASTASSIADES *et al*., 2003; ANASTASSIADES, MAŠTOVSKÁ, LEHOTAY, 2003; LEHOTAY, 2007).

Figure 3 presents a general scheme of the QuEChERS procedure. It begins with a solvent extraction step in which acetonitrile or ethyl acetate or acetone is usually added to the sample. Acetonitrile has superior extraction strength than solvents, enabling a more comprehensive range of pesticide molecules from distinct polarities with proper selectivity and recovery. The next step consists of the salt addition, usually magnesium sulfate and sodium chloride, to induce the separation of the organic aqueous phase through the salting-out effect. Also, the addition of $MgSO_4$ assists water removal from the sample, which increases the recovery of the analytes. Other salts, like sodium nitrate, sodium sulfate, lithium chloride, and even fructose were tested or used on QuEChERS first step (KIM *et al*., 2019).

In the second step, there is a d-SPE process to eliminate matrix interferences. The most common sorbent phase used is a silica phase particle covered of a primary-secondary amine

(PSA), which can chelate effects and remove polar substances, like organic pigments, sugar, and some fatty acids. Octadecylsilane silica particles are commonly employed together with PSA to clean fat content from the samples once it removes non-polar compounds like lipids. Graphitized carbon is also used to take away sterols and pigments (WILKOWSKA, BIZIUK, 2011; REJCZAK, TUZIMSKI, 2015; GONZÁLEZ-CURBELO *et al*., 2015; KIM *et al*., 2019). Despite its simplicity and high recovery of the analytes, QuEChERS does not present good selectivity since it is multiclass/multiresidue; the reason why it is required a separation step to make up this limitation (BRUZZONITI *et al*., 2014).

Figure 3 - General scheme of the QuEChERS procedure



Source: Letícia A. Marques (2021), created with BioRender.com.

Some experimental factors affect QuEChERS extraction such as sample amount, solvent type, sample nature, solvent proportion, salt amount, final pH, water content, *etc*.; so, these parameters might be evaluated and optimized accordingly (REJCZAK, TUZIMSKI, 2015; ŁOZOWICKA, RUTKOWSKA, JANKOWSKA, 2017).

Luzardo and coworkers (2013) published a QuEChERS-based method to determine 57 PCBs and OCPs in human breast milk and colostrum samples. They performed a slight modification on the QuEChERS EN15662 method, followed by GC-MS/MS analysis. Contamination levels for at least 9 of monitored compounds were found, highlighting: PCBs

congeners 138 and 180 had been detected in all samples, and while congener 153 were present in 83% of them; between OCPs, the most present ones were HCB and p,p'-DDE, with quantification of 0.75 and 8.84 μg L^{-1} in colostrum and 0.76 and 9.14 μg L^{-1} in mature milk respectively; DLD, β-HCH, LIND and HCB also showed high contamination levels (LUZARDO *et al*., 2013).

A recent study of 20 breast milk samples from Brazilian mothers was carried out for 16 OCPs and metabolites using a modified QuEChERS procedure followed by GC-MS analysis with negative chemical ionization (NCI). Researchers evaluated formic acid addition to the extractor solvent (acetonitrile), besides using only sodium chloride and magnesium sulfate in the first step of the QuEChERS procedure. A successful validation was performed; however, most samples presented levels of OCPs lower than LOQ values (2.5 to 25 ng g^{-1} lw), which does not demonstrate significant bioconcentration in breast milk (FERRONATO *et al*., 2018).

A combination of Box-Benken design and Plackett-Burman screening design was used to develop an optimized QuEChERS method for 25 pesticides determination in milk samples and dairy products from Turkey. They noticed that sodium acetate amount is the most significant variable of the proposed QuEChERS extraction. After optimization, it was possible validate the analytical method properly (MANAV, DINÇ-ZOR, ALPDOĞAN, 2019).

1.3 New perspectives for PCBs and OCPs analysis

Microfluidics is the scientific field that studies the flowing of minute volumes through micro-sized channels of a well-defined platform. In the latest decade, research published in this field has been increasing expressively due to the significant advances related to the innovations on analytical chemistry, cellular biology, medicine, electronics, *etc*., which can easily fabricate and improve sensibility to the devices. Miniaturized devices are designed to perform complete laboratory analysis of the target compound inside of them, the motive by which they were called micro-total analysis systems (μ-TAS) or "lab-on-a-chip" (LOC). Another essential characteristic of a device with micrometer-sized channels is their portability or wearability, allowing them to carry out fast analysis with minimal handling of the sample. It is possible to highlight advantages as high sensitivity, very-small consumption of reagents and samples (in the order of nanoliters), high throughput analytical frequency, low-cost fabrication, and ease of use (MARK *et al*., 2010; WENG, NEETHIRAJAN, 2017). Optical, mass spectrometry, and electrochemical methods are the most employed microfluidic detection methods (WU, GU, 2011). Electrochemistry can power up microdevice applications due to the miniaturization compatibility, notable sensitivity, low cost, robustness, *etc*. Biosensors are defined as a device

that presents a signal transduction component to generate semi- or quantitative determinations using a biomolecular recognition, which will provide the selectivity (WANG, 2002; RACKUS, SHAMSI, WHEELER, 2015).

Microdevices are a current trend for food residue analysis, such as allergens, toxins, foodborne pathogens, and pesticides. It represents a remarkable improvement for food safety monitoring, which traditionally used expensive and time-consuming sample preparation procedures and or chromatography determinations (ATALAY *et al*., 2011; WENG, NEETHIRAJAN, 2017). However, there are few reports in the literature about microfluidic devices to determine organochlorinated residues in food samples. This section will discuss some interesting papers that describe the fabrication of microchips based on electrochemical detection to PCBs determination in various matrices. It might be seen as an inspiration to the researchers. Once adaptability or slight innovations over cited devices could result in the appropriate usage of chlorinated compounds as PCBs or OCPs determination in milk.

A biosensor based on antibodies for PCB 28 and 77 was used to detect these chlorinated compounds in milk samples. Magnetic beads coupled to protein G were used to immobilize IgG anti-PCB, which was used in a competitive assay with alkaline phosphatase (AP) acting as an enzymatic label competitive site; so, when AP reaches the device substrate produce the electrochemical signal. An arrangement of four graphite electrodes in a low-density screen-printed array was employed as a transducer to generate differential pulse voltammetry (DPV) measurements. The authors analyzed whole and skim milk samples spiked with a PCB standard mixture. First, a multi-step SPE procedure was carried out to remove fat content from milk; the following eluate was dried and reconstituted in methanol or buffer solution and ready to be applied on the electrochemical magneto-immunosensor. An average recovery rate of 80% was obtained, and high sensitivity at sub-ppb levels; equally significant, it was noticed a high reproducibility using the proposed device (CENTI *et al*., 2007).

Recently, Shi and coworkers (2016) developed a photoelectrochemical sensor based on TiO_2 nanorods with molecular imprinting for PCB 101 to detect it. A fluorine-doped tin oxide (FTO) glass was utilized as a substrate for TiO_2 synthesis, performed by the hydrothermal process with simultaneous molecular imprinting using PCB 101 as a template. The entire electrochemical system consisted of the working electrode as the modified TiO_2 matrix, counter electrode of platinum and the reference electrode was saturated calomel electrode; and as irradiation source, a LED lamp (λ = 365 nm; 20 mW cm^{-2}) was used. The working electrode of imprinted TiO_2 nanorods presented a photocurrent density response of 34.1% higher than the non-imprinted one. A wide linear range $8{,}0.10^{-14}$ to $3{,}0.10^{-8}$ mol L^{-1} showed to be linear

(R^2=0.9966); the obtained limit of detection (LOD) was 1,0.10^{-14} mol L^{-1} and recovery rates varied from 96.3 to 101.19% (RSD values were lower than 8.27%). The authors also studied selectivity using PCBs congeners 77 and 126, naphthalene, pyrene, 2,4-D, bisphenol A, and atrazine as interferents, presenting as well good recognition for the target molecule even in the presence of other congeners. Three water samples of Chinese rivers were successfully analyzed using this photoelectrochemical device (SHI *et al*., 2016).

Also, in 2016, a group of Chinese researchers created an electrochemical sensor for PCBs determination using a composite made of β-cyclodextrin polymer (β-CDP), reduced graphene oxide (rGO), polypyrrole (PPy), and with pyrolytic graphite electrode (PGE). Detection was based on a replacement scheme, where the ferrocene molecule acted as a redox indicator. The hydrophobic core of β-CDP/rGO/PPy/PGE includes ferrocene, which the PCB molecule will replace, and then a DPV analysis of ferrocene will be carried out. The composite and the whole platform were properly characterized, and feasibility of the sensor was proved over PCBs determination in sediments from a lake. LOD reached picomolar levels, meaning an ultrasensitive quantification for PCBs; a good reproducibility was obtained (RSD = 3.48%), and it was noticed appropriate device stability during 20 cycles in cyclic voltammetry. A comparative interference study was also performed with eleven substances structurally similar to PCB, and the sensor showed excellent specificity to Aroclor mixtures (ZHENG *et al*., 2016).

It is possible to perform selective electrochemical detection using aptamer, a synthetic oligonucleotide that can promote affinity biorecognition (RACKUS, SHAMSI, WHEELER, 2015). Wu and coworkers developed a biosensor based on an Au electrode modified with a DNA-aptamer to recognize PCB 77. These researchers used EDC-NHS to promote crosslink among Au electrode and ferrocene carboxylic acid. A proper electrochemical characterization of the modified aptamer electrode was carried out with cyclic voltammetry, DPV, and electrochemical impedance spectroscopy (EIS). A linear range from 0.2 to 200 μg L^{-1} and a LOD of 0.01 μg L^{-1} were obtained. A comparison between biosensor and GC measurements of tap water samples spiked with PCB standard indicated an excellent agreement of them, proving that the proposed biosensor is a possibility of it as a reliable point-of-care device (WU *et al*., 2016).

1.4 Conclusions

PCBs and OCPs chemical properties and extensive usage for crops, industrial, and army purposes in the last half-century promoted a long-term contamination since they are persistent organic pollutants. After banishing most of these chlorinated compounds in the '70s, many studies have been developed about their toxicity to animal and human organisms and monitoring their presence in environmental and foodstuff matrices. Some reports described a decrease of PCBs and OCPs levels in human breast milk, while others showed increasing levels, which is a controversial issue highlighting the necessity of continuous surveillance programs worldwide.

Regarding analytical procedures available, classical techniques as LLE to the most modern ones as SPME or QuEChERS have been utilized to carry out PCBs and OCPs extraction and sample preparation, usually followed by GC-ECD, GC-MS or GC-MS/MS analysis. Microfluidic devices based on electrochemical detection are a smart alternative to detect PCBs and OCPs from milk samples simply and cheaply. The use of microdevices is the current state-of-art approach to miniaturization in Analytical Chemistry. Therefore, a significant evolution in terms of the number and quality of published papers relating to the development of microchips for PCBs and OCPs in milk is expected.

CHAPTER 2 – Selection of sample preparation techniques for extraction of PCBs from UHT whole-milk

2.1 Introduction

A chemical analysis includes a series of steps, since sampling, sample handling, processing, measurement or instrumental analysis, followed the data treatment being each step equally important to the final result and must be carried out carefully.

The sample handling includes preliminary processes, as sample weighting or volume measuring, homogenization, size reduction if required, and a set of procedures known as a sample preparation technique (PAWLISZYN, 2003; MOLDOVEANU & DAVID, 2015). In turn, sample preparation involves extracting the analytes, concentration, and/or cleaning-up the sample by interferent removal.

The importance of sample preparation reclines over some facts: the first one is that around 60% of the total time of a chemical analysis is expended on this step, being the most expensive stage once requires a highly qualified professional; the second one, it is the most significant error source of an analytical procedure (KOROL *et al*., 2015; MOLDOVEANU & DAVID, 2015).

Regarding analytes present at trace levels, the sample preparation is more critical than for major components determination; once it might promote appropriate selectivity and the interferents removal can provide a better chromatographic identification, enhancing the analysis performance in quantitative aspects. Besides, there is a variation in speed and convenience of sample prep procedure execution (PAWLISZYN, 2003).

Considering these aspects, analytical chemists have been engaged to improve analysis performance through a well-chosen and executed sample preparation.

2.1.1 Chemometrics

Chemometrics or Cheminformatics is an interdisciplinary field that employs formal mathematical and statistical logic to chemical data to obtain relevant chemical information. Therefore, Chemometrics has improved over the last decades and utilized by many researchers, as engineers, computing scientists, statistics, and chemists. It is possible to state that analytical chemists represent most users of chemometrics tools, once chemometrics is quite beneficial to experimental planning, procedures optimization, data interpretation, or even pattern recognition (BRERETON, 2003; BRERETON *et al*., 2018; FERREIRA, 2019).

There are several specific areas of Chemometrics, among which we might highlight and briefly describe the following ones:

i. Experimental Design: it is a statistical approach to get the best answer possible from a

specific system with a minimum amount of effort and time, representing a great alternative to the traditional tactics of changing one experimental factor at a time. Its usage enables to observe factors interaction and distinguish them in the studied system. This Chemometrics area is also known as Design of Experiments (DoE), and it has been widely applied for screening analysis, optimization of procedures, saving time and resources, and quantitative modeling. There are many experimental designs, such as factorial designs, Plackett-Burmann and Taguchi designs, mixture designs, and response surface designs, as Box Behnken and Doehlert design (BRERETON, 2003; BRERETON *et al*., 2017; BARROS NETO, SCARMINIO, BRUNS, 2010).

i. Pattern Recognition and Multivariate Analysis: this sub-area of Chemometrics accomplishes detecting patterns on the obtained data. There are two main groups of data treatment – the unsupervised pattern recognition and the supervised one. The first group consists of clustering analysis to establish a general correlation between data. In contrast, the second group demands the use of well-known data as a training set, which will provide a mathematical model to classify desired data. The most widespread unsupervised pattern recognition is principal component analysis (PCA), and for supervised analysis is partial least squares (PLS), or artificial neural network (ANN).

ii. Multivariate Calibration: it is the correlation of data sets and independent variables, mainly applicable to near-infrared (NIR), Nuclear Magnetic Resonance (NMR) and MS analysis.

iii. Statistical Methods: basic descriptive statistics calculations, like mean, median, standard deviations, even to significant tests, distributions, confidence intervals, uncertainty, and analysis of variance (BRERETON, 2003; MILLER, 2019).

iv. Digital Signal Processing and Time Series: it refers to raw data transformation through mathematic algorithms or filter usage, being employed mainly for sequential time analysis in chromatography, industrial process control purposes, and spectroscopy, especially for NMR data processing (BRERETON, 2003).

2.1.2 Factorial Design

Factorial designs are easy-to-understand experimental designs and a simple way to the screening of factors. A full factorial design, also known as saturated factorial design, is an approach to identifying the most significant variables for some answers and eliminating those whose are not significant. To perform a typical full factorial design, it is necessary to select *k* factors of interest and *n* levels for each factor evaluated; thus, the total number of experiments

N is defined as $N = n^{k}$ (BRERETON *et al.*, 2017; BARROS NETO, SCARMINIO, BRUNS, 2010).

Factorial designs at two levels are the most used for screening purposes, and they consider all linear interactions between assessed factors. Another aspect of two-level factorial designs is the possibility of working with qualitative parameters, which represents an advantage during a categorical approach. For example, one factor may be a sample dilution (+ level) or not (– level), and another is drying the solution under inert gas or not (+/–, respectively). However, a limitation of the full factorial design is a large number of experiments to carry out if there was a significant number of factors to observe, as is the case of two-levels design for six factors – a total of 64 experiments will be required, which certainly would be a dispendious way to assess interactions from some system (BARROS NETO, SCARMINIO, BRUNS, 2010).

It is also possible to execute a fractional factorial design study, reducing the number of experiments to $n^{(k-b)}$, where b is the fraction and is always less than k. The system achieved information is slightly minimized once the main effects are confounded with interaction terms; however, it is suitable to obtain each effect of the variables by mathematic extrapolating.

Analysis of variance (ANOVA) is a crucial statistical tool to appraise models from designed experiments. Conceptually, it is a hypothesis test based on a comparison of means to determine significant differences between them and if factors of the model influence a dependent variable (BRERETON, 2019).

In experimental design analysis, we must observe the Pareto's chart of effects in which an estimative from standardized effects are plotted versus the effects of the variables. It allows a straight visualization of significant effects of the model, thereby reiterating what had been presented by ANOVA, but sometimes it is hard to identify. For example, each main effect or variables interaction effect is plotted in a bar diagram; so, a bar crossing over *p*-value line, usually defined as 0.05, indicates that it is a significant effect once it has a lower *p*-value than critical *p*-value (WILKINSON, 2006).

2.1.3 Central composite design

Another type of experimental design is the central composite design, based on a two-level factorial design with the addition of k points, where k represents the number of independent variables (BRERETON, 2003; BARROS NETO, SCARMINIO, BRUNS, 2010; MONTGOMERY, 2017; WAGNER JR, MOUNT III, GILES JR, 2014). It is constituted of the following parts:

i. The first one is the factorial (or cubic) part, which has N_f points $x_1 = +1$ or $x_1 = -1$ for all i. They are identical to a two-level full factorial design.

ii. The second portion is an axial (or star) part – it is formed from a total of $2k$ axial points, where each axial point has its coordinates on a set value of ± α and the remaining factors set at level 0. It is important to emphasize that α is defined as $\alpha = (2^k)^{1/4}$ with $|\alpha| \geq 1$.

iii. The third and last part is the central point, in which each factor is taken as 0, *i.e.*, it is $x_1 = \cdots = x_k$, existing asymmetrical design from this point.

The precision of the estimation is influenced by the points position, the number of replicates at the central point, and the set α value.

The central composite design utilization consequence is to estimate the constant term, linear, or quadratic terms, and the interaction between the independent variables. The curvature of the response surface is also estimated since existing central and axial points make it possible. Moreover, the experimental error of the model can be appraised if extra experiments at the central point were carried out (BRERETON, 2019).

Box and Hunter proposed a rotatability definition, which depends on the α value but does not depend on the number of replicates in the central point. It means that the prediction error is the same for all points over the surface response.

A two-factor central composite design is also called star design; in this case, we have $k = 2$, soon $\alpha = \sqrt{2} = 1{,}4142$, and there are three levels assessed for each factor, denoted as $+\alpha$, 0, and $-\alpha$, resulting in nine trials assays and six coefficients to be estimated.

2.2. Aim

This chapter's main objective was to apply chemometric tools to sample preparation step of UHT whole-milk samples to extract PCBs.

2.2.1. Specific objectives

- Evaluate the sample preparation by SPME using a 2^3 full factorial design to PCBs extraction from UHT whole milk.
- Miniaturize the sample preparation step by QuEChERS and perform the greenness assessment of this procedure.
- Evaluate miniaturized QuEChERS procedure using a 2^4 full factorial design and a 2^2 central composite design for PCBs extraction from UHT whole milk.
- Compare SPME and QuEChERS best conditions to determine the most appropriate

sample preparation approach for PBCs extraction from UHT whole-milk samples.

2.3 Experimental

2.3.1 Reagents, solutions, and equipment

A solution of 10 PCBs congeners (28, 52, 101, 118, 138, 153, 156, 170, 180, 189) at 10 µg mL^{-1} of each one was purchased from AccuStandard (New Haven, USA). Magnesium sulfate anhydrous (≥99.5%), sodium chloride (≥99.5%), sodium citrate tribasic dihydrate (≥99.0%), disodium hydrogen citrate sesquihydrate (≥99.0%), Supelclean™ PSA-silica, and cyclohexane (≥99.5%) were obtained from Sigma Aldrich (Saint Louis, USA). Acetonitrile (HPLC grade) was purchased from Honeywell (Raunheim, Germany). Isooctane (HPLC grade) was acquired from Panreac (Darmstadt, Germany). For glassware cleaning, hexane (Panreac - Castellar del Vallès, Spain) and acetone (Synth, Diadema, Brazil) of analytical grade were used.

Solid phase microextraction fibers utilized were: 100 µm polydimethylsiloxane (PDMS); 65 µm PDMS/Divinylbenzene (DVB); 75 µm carboxen/polydimethylsiloxane (CAR/PDMS); 50/30 µm DVB/CAR/PDMS; and 85 µm polyacrylate (PA); all fibers and a fiber holder were acquired from Supelco (Bellefonte, USA). SPME headspace vials (40 mL) with screw cap and PTFE/silicon were acquired from Flow Supply (Cotia, Brazil). A Jacketed beaker, thermostatic bath (Solab, Piracibaba – SP, Brazil), and a magnetic stirrer were also employed to assist the SPME procedure.

2.3.2 Instrumentation

All analyses were performed in Agilent 7890B gas chromatography (GC) equipment with Agilent 7693A autosampler module coupled to a triple quadrupole mass spectrometer Agilent 7010B, located at Federal Laboratory of Animal and Plant Health and Inspection in São Paulo from Ministry of Agriculture, Livestock and Food Supply (MAPA). Separation conditions employed were: two units of HP-5MS columns (15 m × 0.25 mm × 0.25 µm, Agilent); oven start at 60 °C for 1 min, 40 °C min^{-1} until 170 °C; then 10 °C min^{-1} until 310 °C hold for 10 min; injector temperature of 270 °C; 1 µL of injection volume at splitless mode; Helium (N60) flow rate of 0.937 mL min^{-1} at 8.74 psi; backflush system at 1.137 mL min^{-1} and 2.72 psi of pressure. MS conditions used were: +70 eV electron ionization (EI); ion source and interface at 300 °C; Q1 and Q2 quadrupoles kept at 180 °C; transference line at 280 °C; collision cell flow rate at 2.25 mL min^{-1} of He and 1.5 mL min^{-1} of N_2 (N50); acquisition for 20 min at

dynamic multiple reaction monitoring (dMRM); gain factor of 5. Monitored transitions (from precursor to product ions), retention times (RT), collision cell energies, and ion ratio for each analyte are presented in Table 2. These conditions are from a pre-tested method from Agilent Pesticides and Environmental Pollutants Analyzer 4.0, counting with automated retention time locking (Auto RTL) and dynamic MRM. GC-MS/MS data acquisition was made by MassHunter software (Agilent, Santa Clara, USA).

Table 2 - Monitored ions of PCBs congeners analyzed by GC-MS/MS

Analyte	RT (min)	Transitions (*m/z*)		CE (eV)	Ion Ratio
PCB 28	9.04	256.0 → 186.0	(Q)	25	-
(2,4,4'-Triclorobiphenyl)		258.0 → 186.0	(C1)	25	63.0
		186.0 → 151.0	(C2)	25	53.7
PCB 52	9.61	289.9 → 219.9	(Q)	25	-
(2,2',5,5'-Tetrachlorobiphenyl)		255.0 → 220.0	(C1)	10	54.0
		291.9 → 221.9	(C2)	25	63.2
PCB 101	11.12	253.9 → 184.0	(Q)	35	-
(2,2',4,5,5'-Pentachlorobiphenyl)		325.9 → 253.9	(C1)	30	49.4
		325.9 → 255.9	(C2)	30	73.9
PCB 118	12.23	325.9 → 255.9	(Q)	30	-
(2,3',4,4',5-Pentachlorobiphenyl)		325.9 → 253.9	(C1)	30	66.9
		327.9 → 255.9	(C2)	30	63.5
PCB 153	12.62	287.9 → 217.9	(Q)	40	-
(2,2',4,4',5,5'-Hexachlorobiphenyl)		359.9 → 289.9	(C1)	25	81.9
		361.9 → 289.9	(C2)	25	52.2
PCB 138	13.12	359.9 → 289.9	(Q)	30	-
(2,2',3,4,4',5'-Hexachlorobiphenyl)		287.9 → 217.9	(C1)	40	85.2
		361.9 → 289,9	(C2)	30	68.3
PCB 156	14.02	359.9 → 289.9	(Q)	25	-
(2,3,3',4,4',5-Hexachlorobiphenyl)		287.9 → 217.9	(C1)	35	93.1
		361.9 → 289.9	(C2)	25	60.7
PCB 180	14.30	393.8 → 323.8	(Q)	30	-
(2,2',3,4,4',5,5'-Heptachlorobiphenyl)		393.8 → 358.8	(C1)	15	59.2
		395.8 → 325.8	(C2)	15	64.9
PCB 170	14.82	393.8 → 323.8	(Q)	30	-
(2,2',3,3',4,4',5-Heptachlorobiphenyl)		393.8 → 358.8	(C1)	15	81.4
		358.8 → 323.8	(C2)	15	59.7

Legend: RT – retention time; CE – collision energy; Q – quantification ion; C1 and C2 – confirmation ions 1 and 2, respectively.

2.3.3 Selection of SPME fibers

Five different commercial fibers (100 µm PDMS; 65 µm PDMS/DVB; 75 µm CAR/PDMS; 50/30 µm DVB/CAR/PDMS; 85 µm PA) were employed to investigate the best

selectivity to PCBs extraction from milk samples. Each fiber was previously conditioned according to the manufacturer's instructions. Five mL of milk samples spiked with PCBs standard solution to generate a 5 ng mL^{-1} of analytes in the sample bulk were contained in a 40 mL vial. Extraction and desorption conditions for SPME fibers selection were defined arbitrarily: 10 min of pre-equilibrium time; headspace extraction at 55 °C for 50 min; thermal desorption at 270 ºC for 10 min. SPME procedure was carried out in triplicate to each fiber, and a clean-up step between each procedure was performed by fiber exposure at 270 °C at 20 min to avoid carryover of fibers.

2.3.4 Matrix of 2^3 factorial design for SPME to extract PCBs from UHT whole milk

Following fiber selection, we delineated a 2^3 factorial design (Table 3) to observe if experimental variables (NaCl concentration, temperature, and time) significantly affected the PCBs extraction from milk.

Table 3 - Matrix of 2^3 factorial design for SPME to extract PCBs from UHT whole-milk

Variables	**Levels**		
	−1	**0**	**1**
X_1 = NaCl concentration (% w/v)	0	18	36
	−1	**0**	**0.615**
X_2 = Extraction temperature (°C)	70	83	91[a]
	−1	**0**	**1**
X_3 = Extraction time (min)	30	60	90

Legend: [a] Due to experimental limitations, we used 91 ºC (X_1=0.615) as extraction temperature instead of 96 ºC (X_1=1) for the symmetry of the 2^3 factorial design.

2.3.5 Miniaturized QuEChERS procedure and experimental designs approaches

QuEChERS extraction conditions used were miniaturized and adapted from Luzardo *et al.* (2013); then, the significant factors were optimized by 2^4 factorial design followed by a 2^2 central composite design. To proceed with the sample preparation, 1 mL of UHT milk spiked with PCBs standard solution to provide 5 ng mL^{-1} of analytes in the sample was placed into a 15 mL Falcon™ tube. Then was added 1 mL of ultrapure water, 2 mL of acetonitrile, and 1.3 g of a mixture of magnesium sulfate ($MgSO_4$), sodium chloride (NaCl), sodium citrate tribasic dihydrate ($Na_3C_6H_5O_7 \cdot 2\ H_2O$), and disodium hydrogen citrate sesquihydrate ($Na_2C_5H_6O_7 \cdot H_2O$) salts in a ratio of 4:1:1:0.5, respectively (mixture of QuEChERS salts 1 - MSQ1). A vortex homogenization was made for 30 seconds for each tube, followed by centrifugation (5 min at 2580 RCF). After the first step, the supernatant was removed, and a re-extraction step was

performed by adding 1 mL of acetonitrile and executing agitation-centrifugation stages once more. The collected fractions were transferred to another 15 mL Falcon ™ tube containing 280 mg of a mixture of 100 mg of PSA and 180 mg of $MgSO_4$ anhydrous (called mixture of QuEChERS salts 2 - MSQ2) to proceed d-SPE step. A new agitation-centrifugation was carried out, and then 2.5 mL of extract was transferred to a test tube. Next, we executed a drying step under slight N_2 flow until dryness. Finally, the residue was dissolved in 500 µL of cyclohexane and injected into GC system.

To verify which experimental variables are significant to the miniaturized QuEChERS procedure, a 2^4 factorial design was planned to variables – sample volume (X_1), MSQ1 amount (X_2), PSA amount (X_3), and solvent volume (X_4) (Table 4).

Table 4 - Matrix of 2^4 factorial design for the miniaturized QuEChERS to extract PCBs from UHT whole-milk.

Variables	**Levels**		
	-1	0	1
X_1 = Sample volume (mL)	0.5	1.0	1.5
X_2 = MSQ1 amount (g)	0.65	1.30	1.95
X_3 = PSA amount (mg)	50	100	150
X_4 = Solvent volume (mL)	2	3	4

From data of 2^4 factorial design, a walking over the model surface was delineated trying to achieve a maximum point. Steps (Δ) for the walking were obtained by regression coefficients ratio of two significant variables (X_1 and X_2) from the 2^4 factorial design. Then, a series of experiments were performed, including central point (CP) and from CP + 1Δ to CP+5Δ. Following, experimental conditions of CP + 1 Δ essay (*i.e.*, 1425 µL of sample volume and 4000 µL of MeCN) was taken as basis for the 2^2 central composite design central point establishment, with slightly adjustment of sample volume.

So, the central composite design (CCD) 2^2 delineated for the variables - sample volume (X_1) and solvent volume (X_2) are presented in Table 5.

Table 5 - Matrix of central composite design 2^2 for the miniaturized QuEChERS to extract PCBs from UHT whole milk.

Variables	**Levels**				
	-1.4142	-1	0	+1	+1.4142
X_1 = Sample volume (µL)	1117	1200	1400	1600	1683
X_2 = Solvent volume (µL)	3434	3600	4000	4400	4566

2.3.6 Data analysis

Chemometrics data were evaluated using Statistica™ 10 (StatSoft Incorporation) software and Microsoft Excel (Microsoft Corporation, Redmond - WA, USA) was used for previous data tabulation.

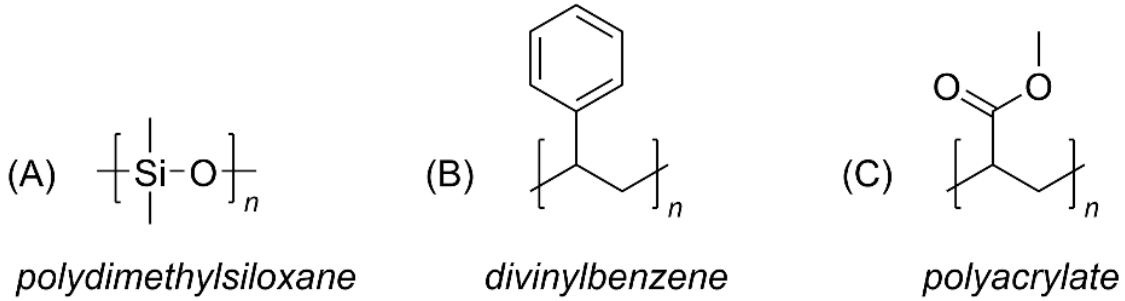
2.4 Results and discussion

2.4.1 Results of SPME fibers selection and factorial design

A proper SPME fiber selection is important to achieve the most efficient extraction-desorption cycle possible. This process depends on several characteristics of the analyte, as molecular weight and size, polarity, type of functional groups, vapor pressure, and concentration (MANI, 1999; SPIETELUN *et al*., 2010).

There are many options of coatings for SPME fibers, some of them made of sorbents phases commercially available, and others based on new sorbent coatings synthesis. In this work, we employed five SPME fibers of distinct coatings. Figure 4 shows the chemical structures of the monomer for each polymeric phase of used fibers.

Figure 4 - Chemical structures for polymeric coverings of use SPME devices



Source: MARQUES (2021), adapted from SHIREY (2012).

It is possible to classify commercial coatings into two categories: homogenous coatings and porous particles embedded in a polymeric film. The first group includes one-phase made fibers, as PDMS and PA. Both will retain analytes by absorption. Although these two fibers present the same retention mechanism, they are distinct in polarity – PA is polar, while PDMS is nonpolar. Commercial polyacrylate fibers have 85 µm of thickness, being partially crosslinked. The nonpolar PDMS coating is one of the most commonly utilized sorbent phase for SPME because of its characteristics. First, the beginnings of SPME as a sample prep technique and theoretical fundamentals beginning were established using PDMS fibers.

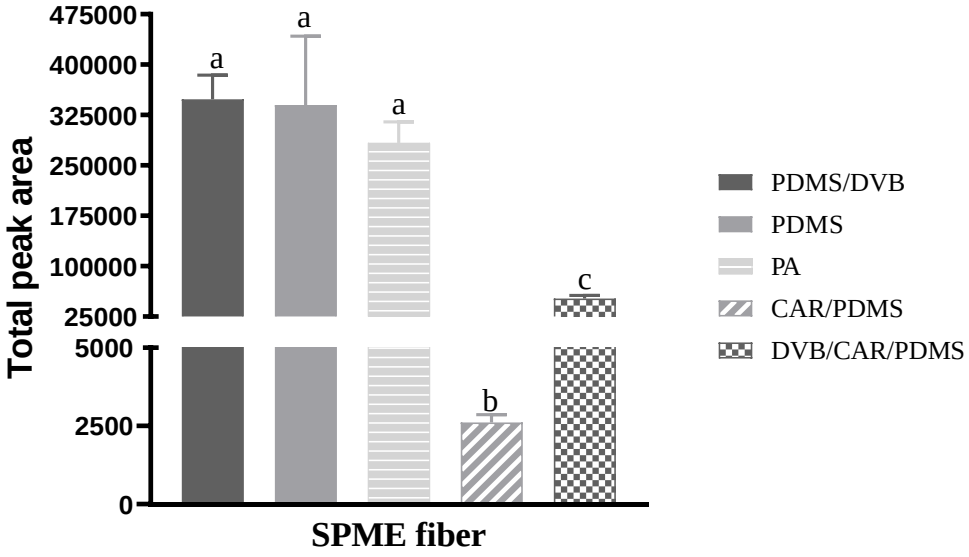
Second, it is also a classic GC stationary phase since polydimethylsiloxane polymer has thermal stability up to 300 °C. Furthermore, their decomposition products are well-known and easy to identify at MS. Three commercial thickness of PDMS fibers are available – 100 and 30 µm, both made of nonbonded coatings; and 7 µm, made of bonded coating, having higher thermal stability (up to 340 °C) (MANI, 1999; SPIETELUN *et al*., 2010).

The other fibers group comprises of a mixture of particles blended on a polymeric phase, which were partly crosslinked. This group is allocated PDMS/DVB, CAR/PDMS, and DVB/CAR/PDMS fibers. These three fibers are bipolar and therefore, retention will be different based on the combination of each material coating (SHIREY, 2012).

DVB is a solid polymer particle with surface area of 750 $m^2\ g^{-1}$. Its particles are mainly mesopores, which promote the physical retention of analytes that fit into the pore. Thus, PDMS/DVB fibers can retain smaller analytes than the fiber of PDMS pure. Carboxy is also porous polymer particles, presenting around 2 µm of particle size, 715 $m^2\ g^{-1}$ of surface area, 0.78 mL g^{-1} of total pore volume with an equality distribution amongst macro, meso, and micropores. The CAR/PDMS fiber has an increased surface area over PDMS since CAR was added to its composition (MANI, 1999).

Next, we will proceed to the obtained results for the selection of SPME fibers. Figure 5 shows the average total peak area of the 10 PCBs monitored for each fiber *(n=3)*.

Figure 5 - Results of total peak area for extraction (*n=3*) of PCBs (5 ng mL^{-1}) from UHT whole milk using different SPME fibers.



Legend: The same letter indicates that there is no significant difference between two averages by ANOVA and Tukey tests (α = 0,05).
Source: Letícia A. Marques (2021).

We observed that PDMS, PDMS/DVB, and PA fibers presented extraction efficiency without significantly difference (α=0.05). For PDMS fibers, this result is explained due to Van der Waals interactions established between PCBs and the extractor phase. Regarding PDMS/DVB fibers, which present mixed behavior, it is possible that extraction of PCBs could be favored by the sum of interactions Van der Waals forces and π-π interactions between divinylbenzene and biphenyl core of PCBs (KUDLEJOVA, RISTICEVIC, VUCKOVIC, 2012). An interesting result for PCBs extraction from milk was achieved using PA fiber, a moderately polar coating. Despite non-polarity of PCBs, the extraction efficiency from PA fiber is explained by its high affinity to aromatic compounds and oxygenated analytes (SHIREY, 2012).

For this study, the smaller values of PCBs total area were achieved using DVB/CAR/PDMS and CAR/PDMS fibers. We noticed a decreased response for CAR/PDMS fiber compared to the triple-phase fiber results, which is expected since CAR/PDMS does not present aromatic groups as divinylbenzene from the triple-phase fiber to improve PCBs retention into polymeric bed.

Finally, there is no significant difference (α = 0,05) for total peak area values of PCBs extracted using PDMS, PA, and PDMS/DVB fibers by ANOVA and Tukey test. However, a higher RSD value (29.95%) was obtained when PDMS fiber was employed compared to 10.17% for PDMS/DVB and 10.98% for PA fibers. Then the 65 µm PDMS/DVB fiber, which presented the lowest RSD value, was chosen for further experiments once it promotes better precision.

Table 6 – Results for matrix of 2^3 factorial design matrix for PCBs extraction from milk and its response function

Run	**Variable**			**Response function**
	NaCl concentration	*Temperature*	*Time*	Total peak area
1	−1	−1	−1	764218
2	1	−1	−1	70472
3	−1	0.615	−1	1407120
4	1	0.615	−1	4412800
5	−1	−1	1	5566376
6	1	−1	1	2078325
7	−1	0.615	1	7841306
8	1	0.615	1	17322313
9	0	0	0	3792753
10	0	0	0	3204909
11	0	0	0	3036770

Results for 2^3 factorial design for PCBs extraction using PDMS/DVB fiber are presented in Table 6. From these data, it was possible to obtain a model described by Equation 14.

$$y = 4326272 + 1038111\,X_1 + 2668412X_2 + 3269214\,X_3 + 2083561\,X_1X_2 + 460128\,X_1\,X_3 + 1566711\,X_2X_3 + 1158704\,X_1X_2X_3 \qquad R^2 = 0.9509 \qquad \textit{Equation 14}$$

Where y is the total peak area of 10 analytes, X_1 is NaCl concentration (% w/v), X_2 is the temperature (° C), X_3 is time (min), and R^2 is the determination coefficient.

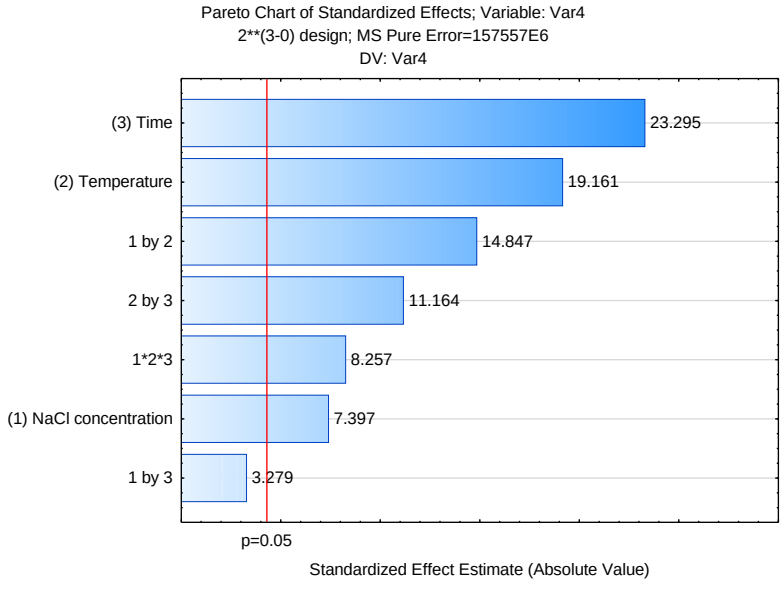
ANOVA for the 2^3 factorial design is presented in Table 7.

Table 7 - ANOVA for the 2^3 factorial design matrix of PCBs total peak area extracted from UHT whole milk using PDMS/DVB SPME fiber

Variable	Sum of squares	D.f.	Mean square	F-value	*p-value*
(1) NaCl concentration	8.62E+12	1	8.62E+12	54.719	0.018
(2) Temperature	5.78E+13	1	5.78E+13	367.145	0.003
(3) Time	8.55E+13	1	8.55E+13	542.674	0.002
1 by 2	3.47E+13	1	3.47E+13	220.427	0.005
1 by 3	1.69E+12	1	1.69E+12	10.750	0.082
2 by 3	1.96E+13	1	1.96E+13	124.632	0.008
1*2*3	1.07E+13	1	1.07E+13	68.171	0.014
Lack of Fit	1.10E+13	1	1.10E+13	69.566	0.014
Pure Error	3.15E+11	2	1.58E+11		
Total SS	2.30E+14	10			

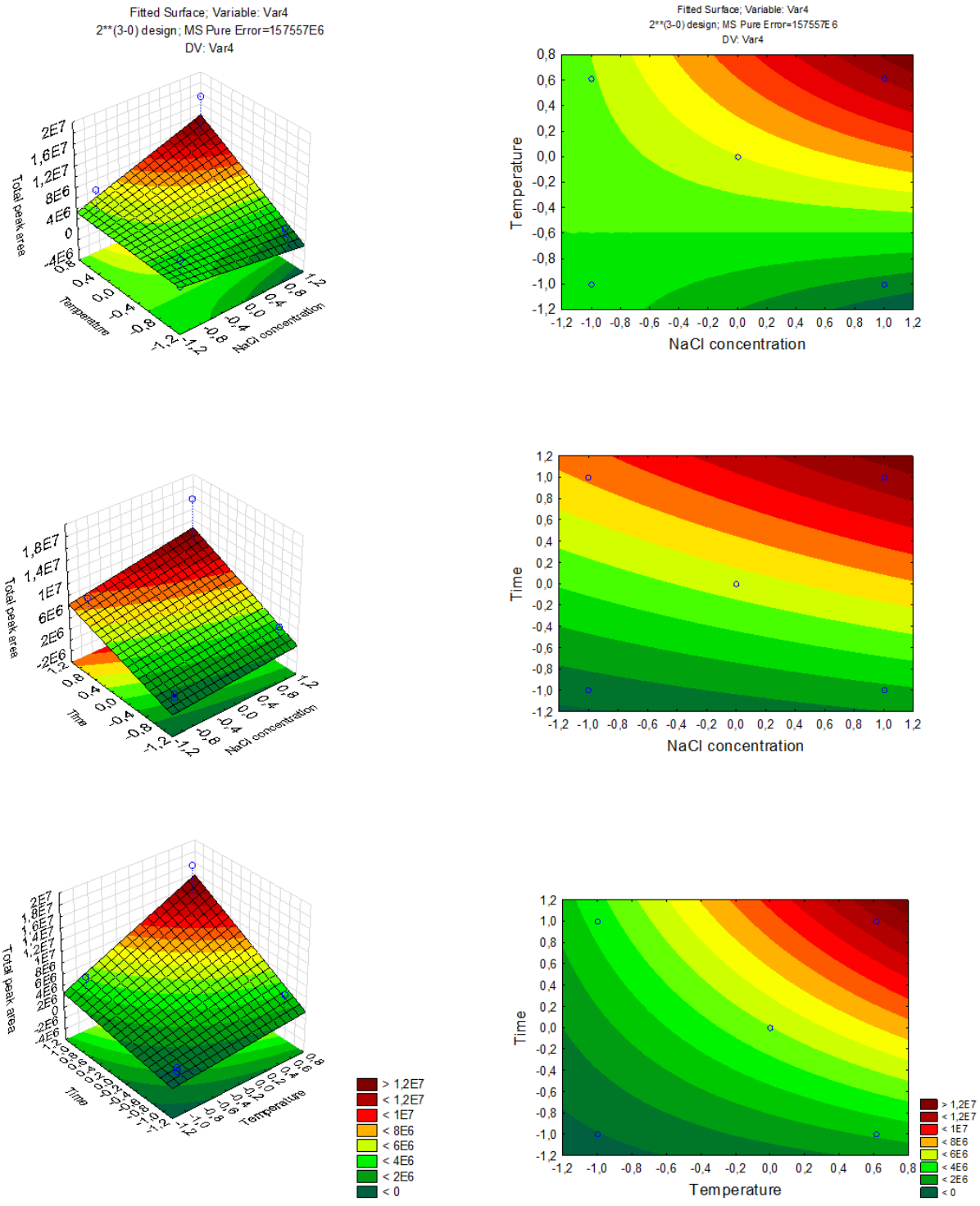
Legend: D.f. = degrees of freedom.

Figure 6 - Pareto's chart for the 2^3 factorial design matrix of PCBs total peak area extracted from UHT whole milk using PDMS/DVB SPME fiber.



Source: Letícia A. Marques (2021).

Figure 7 - Response surfaces and contour plots for 2^3 factorial design matrix of PCBs total peak area extracted from UHT whole milk using PDMS/DVB SPME fiber.



Source: Letícia A. Marques (2021).

We noticed that all variables were significant for the proposed model, and their interaction, with an exception for X_1X_3 interaction, which was not significant at 95% confidence level. Pareto's chart (Figure 6) illustrates these results. On the other hand, we can also check

that F value for lack of fit (69.566) is higher than F critical value (9.0), so the model present lack of fit. It means that the obtained mathematical modeling cannot be used to perform predictions. Observed residues showed to be randomly dispersed (vide Figure A 1); then we can affirm that there are no tendencies for this model. It is also relevant to observe that the model presented a lack of fit, which means that we could not use it for mathematical predictions.

Then, the best condition achieved for 2^3 factorial design for SPME extraction of PCBs is described by $X_1 = X_3 = 1$ and X_2=0.615, corresponding to the following conditions: 36% w/v for NaCl concentration, 91 °C for temperature, and 90 min for extraction time.

2.4.2 Miniaturization of QuEChERS and its evaluation as a green method

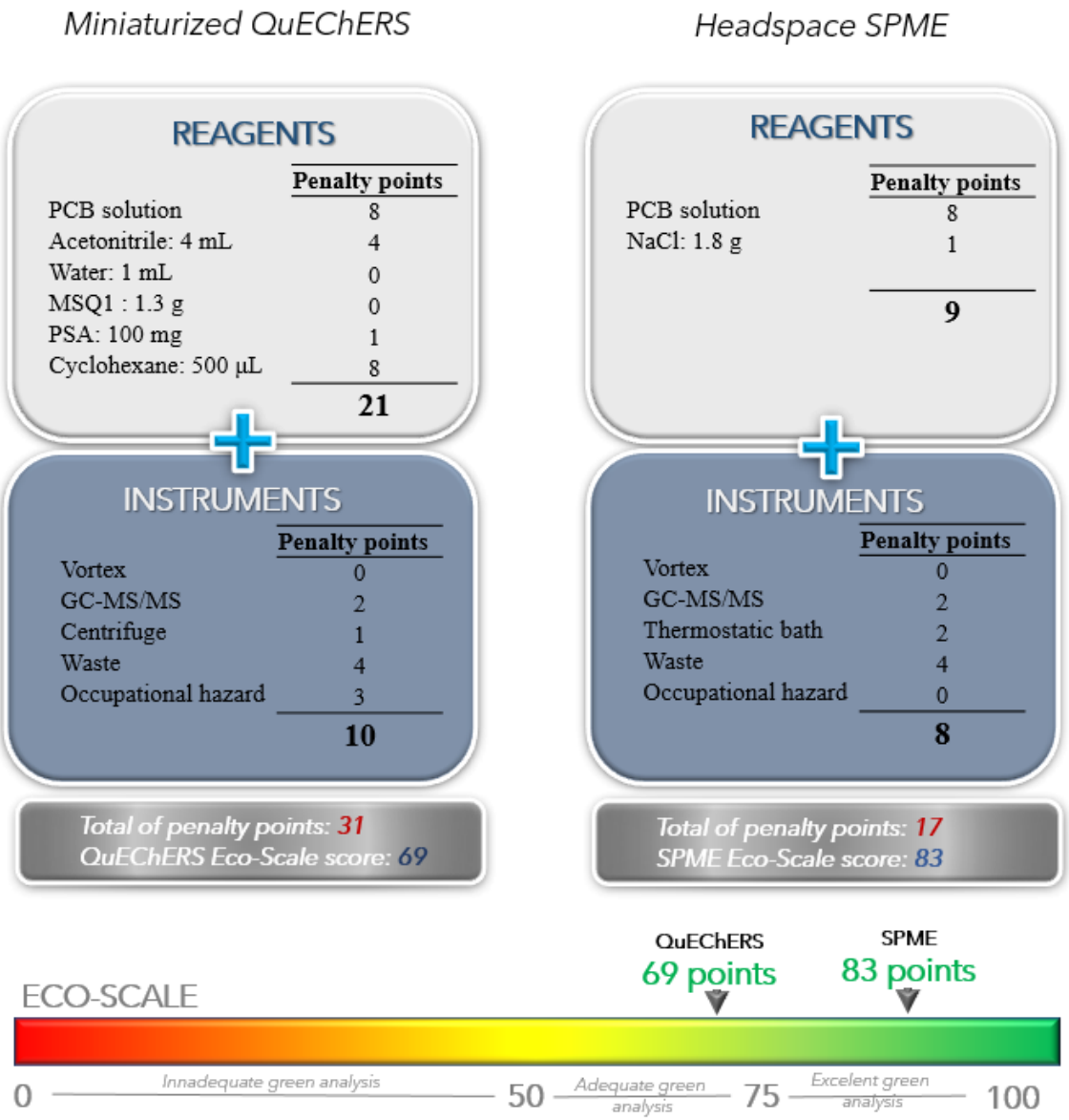
We decide to miniaturize QuEChERS procedure proportions to follow some principles of Green Analytical Chemistry (SHELDON, 2018), as waste prevention and analytical methods for pollution prevention, and minimizing costs for each determination. Current trends on analytical methods have been conceived to attend Green Chemistry precepts (ARMENTA, GARRIGUES, DE LA GUARDIA, 2015; KORANY *et al*., 2017). In this way, sample preparation through miniaturized QuEChERS contemplates important Green Chemistry principles as reducing the amount of sample and reagents, less solvent consumption, minimized waste generation.

Both tests for spiked samples (0.05 and 5.00 ng mL^{-1}) showed a total peak area reduction on a factor of 2.7 and 3.8 times, respectively, if compared with the conventional proportions of the QuEChERS. Thus, a suitable RSD% was observed for the two-level sample batches tested, and the total area decreasing does not imply an evident lack of sensibility. Moreover, we considered an outstanding commitment to this slight sensibility diminution but enabling cost reductions and elaborating a greener methodology.

Many papers are discussing how green metrics should be assessed, mainly that applied to organic synthesis, *e.g.*, carbon efficiency, reaction mass efficiency (RME), atom economy (AE), *etc*. (SHELDON, 2018). For analytical procedures, there are a couple of approaches to green chemistry assessment. The oldest one is the National Environmental Methods Index (NEMI), a famous free database hosted on the internet (KORANY *et al*., 2017), which contains analytical methods evaluated according to four criteria – PBT (persistent, bioaccumulative, and toxic), Hazardous, Corrosive, and Waste. Despite establishing these criteria, NEMI labeling is considered a qualitative tool to perform greenness metrics and does not consider energy consumption over the method execution (KEITH *et al*., 2007; TOBISZEWSKI *et al*., 2015). Therefore, we consider a scale a better way to appraise greenness metrics.

We used the scale known as Eco-Scale proposed for Gałuska and co-authors (GAŁUSKA *et al*., 2012) to estimate how green our procedure is. The Eco-Scale ranges from 0 (inappropriate green method) to 100 points (excellent green method), obtained from the discount of penalty points of the maximum pointing. Penalty points are used to a proper greenness assessment, attributed to the amount of reagents and solvents used per sample, occupational hazard, the energy of instruments spent per sample analysis, and even transportation or sample preservation (see more details in Section A1 from Appendix A) (GAŁUSKA *et al*., 2012; KEITH *et al*., 2007). Figure 8 shows the summarized calculations of penalty points for both sample preparation techniques evaluated in this chapter.

Figure 8 - Assessment of Eco-Scale for the proposed procedures - miniaturized QuEChERS procedure and headspace SPME



Source: Letícia A. Marques (2021).

The final score for the proposed QuEChERS method is 69, considered an acceptable green method. Nevertheless, when using Eco-Scale to assess the greenness of the optimized SPME procedure, we obtained a total score of 83 points. There is a noteworthy reduction in penalty points of our optimized headspace SPME procedure compared to miniaturized QuEChERS method, once is free of organic solvent and occurs in a closed system.

Moreover, we were able to increase 9 points on the Eco-Scale from the original procedure proposed by Luzardo *et al*. (2013) just by performing the miniaturization and optimization of the method. Besides being a more environmentally friendly procedure, our QuEChERS miniaturization presents a remarkable decrease of the cost per analysis: it was achieved a 66.7% of reagents cost reduction for each determination (approximately US$1.05 per analysis).

2.4.3 Experimental designs for miniaturized QuEChERS procedure

Once miniaturization exhibited analytical sensibility, we planned a 2^4-factorial design for sample volume (X_1), the weight of MSQ1 (X_2), weight of PSA (X_3), and volume of acetonitrile (MeCN) added (X_4). Data is presented in Table A2, as well Equation A1 describe the mathematic model obtained for this experimental design.

We noticed that just linear effects of X_1 and X_4 and the linear interaction of X_1 and X_4 were significant ($p < 0.05$). In terms of analysis cost, the non-significance effect of X_3 (PSA weight) is interesting since we required a smaller amount of this expensive reagent. ANOVA shows no lack of fit from the model (Table A3); however, its curvature was not significant at 95% of confidence, indicating that chosen levels to our factorial design do not include a maximum point. Pareto's chart for the 2^4 factorial design obtained model may also be accessed in Figure A 2.

Then we walked straightforward over the model surface (central point – CP; CP + 1Δ to CP+5Δ) according to Barros Neto, Scarmino & Bruns (2010) directions, to figure out a maximum point. Data are presented in Table A4 (Appendix A). Increasing values of total peak area were obtained when moving forward over the surface (*i.e*., enhancing steps). We found out that CP+2 Δ essay is a critical condition to clean-up (vide Figure A3). In addition, after injection of this samples set, GC inlet liner was found visibly dirty. Then, a good compromise between an appropriate clean-up to the sample preparation step, the convenient lifespan of consumable parts and the equipment itself, and repeatability (RSD of 7.3%) were obtained at the CP+1Δ essay. So, we consider CP + 1Δ conditions (where $X_1 = 1.70$ and $X_4 = 2.0$, it means 1425 µL of

milk sample and 4 mL of acetonitrile) to delineate an approximated central point for the 2^2 central composite design (as described previously in Table 5).

Table 8 presents data obtained for the 2^2 central composite design for the miniaturized QuEChERS procedure. This last approach presents a polynomial model (Equation 15) with significant curvature. ANOVA showed at Table 9 indicates that the effects of linear X_1 and square X_2 were significant ($p < 0.05$). Pareto's chart (Figure 9) summarizes data of ANOVA of the 2^2 central composite design.

$$y = 314474{,}6 + 64296{,}8\,X_1 - 4244{,}9\,X_1^2 - 1963{,}5\,X_2 - 84514{,}8\,X_2^2 + 38807{,}3\,X_1X_2$$

R^2 = 0.5946 *Equation 15*

Where *y* is the total area of analytes, X_1 is the sample volume (µL), X_2 is the solvent MeCN volume (µL), and R^2 is the determination coefficient.

Table 8 - Results for matrix of 2^2 central composite design for the miniaturized QuEChERS to extract PCBs from UHT whole-milk

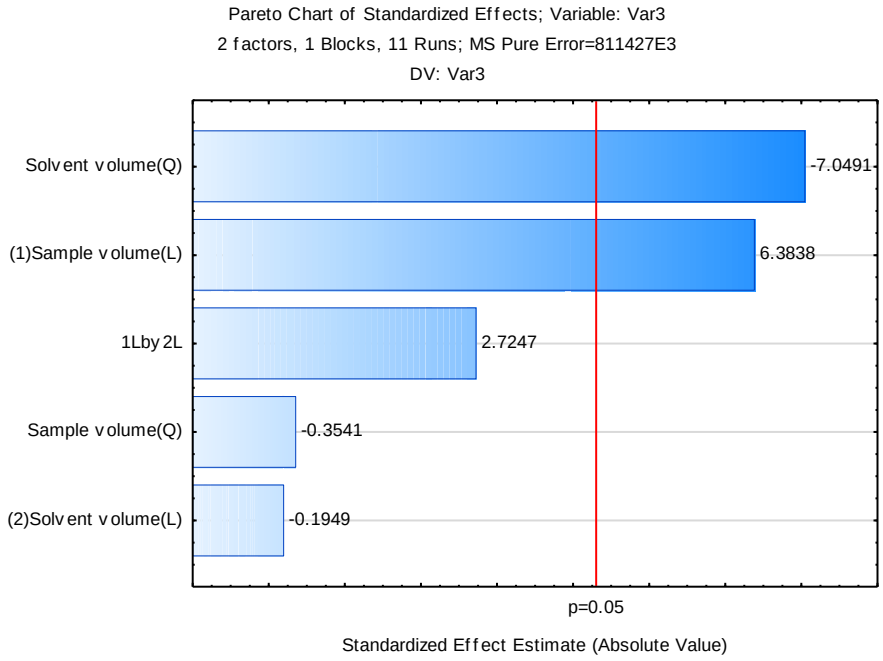
Running	Variable		Response function
	Sample volume	MeCN volume	Total peak area
1	-1	-1	216567.26
2	1	-1	124675.88
3	-1	1	181354.37
4	1	1	244692.11
5	0	-1.414	214939.35
6	0	1.414	143858.15
7	-1.414	0	147934.58
8	1.414	0	531845.66
12	0	0	282613.78
14	0	0	337508.09
15	0	0	323261.02

Table 9 - ANOVA for the 2^2 central composite design obtained for the miniaturized QuEChERS to extract PCBs from UHT whole-milk

Variable	SS	df	MS	F	*p*-value
(1)Sample volume (L)	3.307E+10	1	3.307E+10	40.752	0.024
Sample volume (Q)	1.017E+08	1	1.017E+08	0.125	0.757
(2)Solvent volume (L)	3.084E+07	1	3.084E+07	0.038	0.863
Solvent volume (Q)	4.032E+10	1	4.032E+10	49.689	0.020
1L by 2L	6.024E+09	1	6.024E+09	7.424	0.112
Lack of fit	5.432E+10	3	1.811E+10	22.313	0.043
Pure error	1.623E+09	2	8.114E+08		
Total SS	1.380E+11	10			

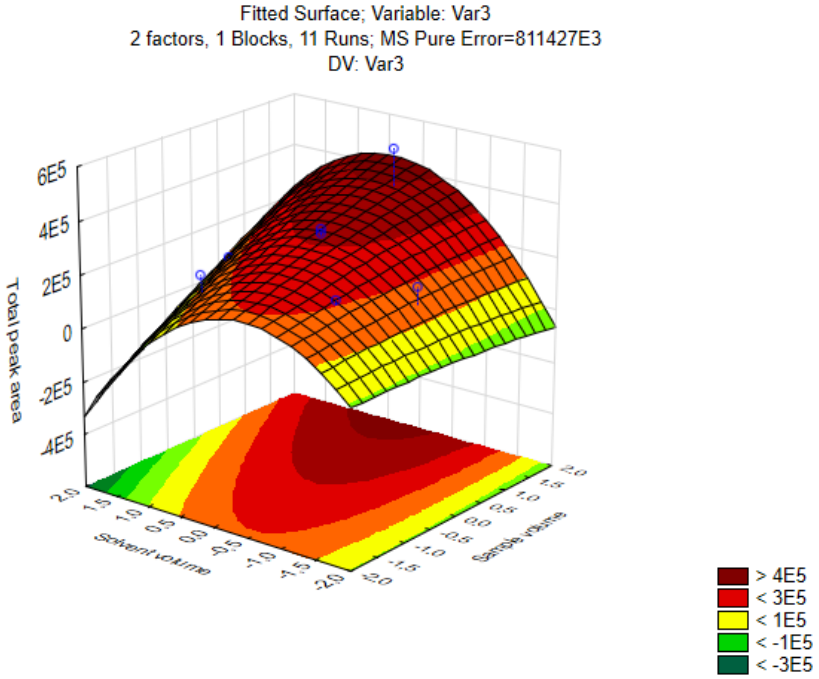
Legend: D.f. = degrees of freedom.

Figure 9 - Pareto's chart of the 2^2 central composite design obtained for the miniaturized QuEChERS to extract PCBs from UHT whole-milk



Source: Letícia A. Marques (2021).

Figure 10 - Response surface of the 2^2 central composite design obtained for the miniaturized QuEChERS to extract PCBs from UHT whole-milk



Source: Letícia A. Marques (2021).

Finally, the best conditions for our miniaturized QuEChERS sample preparation for PCB are given by X_1 = 1.4142 and X_2 = 0.0, *i.e.*, 1683 μL of milk sample and 4000 μL of MeCN solvent). Response surfaces for the 2^2 central composite model are available in Figure 10. So, we used these conditions to perform analytical validation of the method. To facilitate the experimental procedure, a quite simple modification was done: the optimal sample volume (1683 μL) was changed to the nearest approximate value (1700 μL) without compromising the extraction process's good repeatability.

2.4 Conclusions

In this chapter, we observed a difference in performance between these two sample preparation techniques used to extract PCBs from UHT whole-milk samples. In fact, SPME best conditions presented higher values of the total area than miniaturized QuEChERS performed at its best extraction conditions. However, in terms of analytical frequency, it is not viable to carry out an analytical validation using manual SPME extraction since the optimum condition for the time was 90 min. Once this sample preparation represents an expressive enhancement of the sensibility on the residue analysis, we highly recommend SPME usage in case the lab has facilities at the lab that turn possible to do automatized SPME procedures CTC Combi PAL autosampler.

On the other hand, the miniaturized QuEChERS procedure showed to be a proper alternative for PCBs extraction, presenting advantages as greener analysis than the original size method, cost reduction, and a higher analytical frequency with the best conditions obtained from the experimental design, which certainly is crucial to perform analytical validation of the proposed method.

CHAPTER 3 – Validation of a sample preparation method and assessment of PCBs contamination on UHT whole-milk samples from São Paulo state, Brazil

3.1 Introduction

As the control of contaminants residues in foodstuff represents a crucial matter regarding food safety, it is indispensable that the methods for its determination present high reliability. A food contaminant is defined as a compound that, when found in the foodstuff matrix, turns the consumption of this aliment unsafe for human beings. Food contaminants can be classified as follow: *(i)* avoidable, having no safe concentration levels to be ingested; or *(ii)* unavoidable, when permissible levels are set pondering health risks to its exposure and unavoidable formation of such contaminant (PATEL, MILLER, 2012).

There are three primary food contamination sources – microbiological, chemical, and physical – and those resulting from a cross-contamination nature (AIFS, 2021). Food contamination occurs through the food matrix exposure through in several ways: environmental contamination, animal feeding contamination, treatment of animal diseases, or during food conditioning, processing, packing, transport, storage, and distribution. We can mention as examples of food contaminants mycotoxin, packing materials, radionuclides, food additives, heavy metals, dioxins, pesticides, endocrine disruptors, and veterinary drug residues (antibiotics, anti-parasitic, anti-inflammatory, anabolics, *etc*.) (NIELEN, MARVIN, 2008; HUSSAIN, 2016; ALEXANDRE *et al*., 2019).

Regulatory bodies have crucial importance for national and international purposes to ensure food safety, impacting directly over a foodstuff supply chain and commercial trade balance. A regulatory agency is responsible for setting up standards and enforces safety over some area or aspect, with regulatory or supervisory activities. Good performance of regulatory agencies may avoid contamination and misbranding or adulteration of food or other adverse events that can lead to public health problems, as a foodborne pandemic disease dissemination. For instance, if producers of poultry meat from country A eventually had contamination with Salmonella pathogen. They try to export it to country B, the regulatory agency of country A would detect the contaminant, block this exportation, and look out for eliminating the source of the pathogen in country A; otherwise, the export cargo would arrive in the country B where the local regulatory agency would detect the pathogen and block the import (PATEL, MILLER, 2012; ALEXANDRE *et al*., 2019).

In addition, regulatory bodies have been providing guidelines about analytical method validation, which is currently seen as part of developing and establishing of analytical methods (ARAUJO, 2009). For example, any new analytical technique used for food contaminants residues quantification must go through an analytical validation procedure.

3.1.1 Analytical validation

The validation of an analytical method comprises many parameters determined to demonstrate that such a method is consistent, reliable, and accurate, which means it is proper for quantitative purposes. For example, in residues analysis, validation has a crucial role in assessing if the method is suitable for the MRL values established for such contaminants. A typical validation comprises the determination of figures of merit as selectivity, linearity, dynamic range, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, and robustness (PERIS-VICENTE, ESTEVE-ROMERO, CARDA-BROCH, 2015, INMETRO, 2016).

Selectivity is defined as the capacity of the method to distinguish the signal of the analytes from the baseline or interferent signals. It is a fundamental parameter, and good selectivity is required for both qualitative and quantitative purposes. Toward to chromatographic analysis, a method is considered selective if the analyte is resolved, *i.e.*, the blank chromatograms do not present peak or baseline distortions near to RT of analyte and interferences do not overlap with the analyte signal (PERIS-VICENTE, ESTEVE-ROMERO, CARDA-BROCH, 2015; MARSON *et al.*, 2020).

Linearity is the parameter of the method that demonstrates how much an analytical answer *y* is proportional to a variation in the concentration *x*. The mathematic model that describes linearity is called the calibration curve or analytical curve, represented by $y = a\,x + b$, where *b* is the linear coefficient (or intercept), and *a* is called angular coefficient (or slope). Analytical curves are generally obtained from a linear regression of data, and the most common regression method is ordinary least squares (OLS), which is pertinent to homoscedastic data. When a data set is heteroscedastic, the linear regression parameters should be estimated using weighted least squares (WLS). Whatever the type of regression method employed, it is always possible to attribute weight to the regression to obtain the best fit of data, such as $1/x$, $1/x^2$, $1/y$, or even logarithmic weight (ALMEIDA, CASTEL-BRANCO, FALCÃO, 2002; INMETRO, 2016; MARSON *et al.*, 2020).

The range is the interval between minimal (lower) and maximum (upper) concentrations that can be determined with proper precision, accuracy, and linearity. An analytical range is wider than a linear range since the first one contains the lowest concentration (limit of detection) to the highest concentration. Usually, a range varies from 50 to 150% of the supposed

concentration or MRL in the real sample (ARAUJO, 2009; PERIS-VICENTE, ESTEVE-ROMERO, CARDA-BROCH, 2015).

Limit of quantification (LOQ) is defined as the lowest concentration capable of being quantified with acceptable accuracy and precision. In many methods, the lower concentration of the analytical curve is taken as LOQ when for this level were obtained proper precision and accuracy for at least six replicates (PERIS-VICENTE, ESTEVE-ROMERO, CARDA-BROCH, 2015; INMETRO, 2016; MARSON *et al*., 2020).

Precision is the proximity between values obtained by replicate measurements from a quantity under specified conditions. Assessment of precision includes intra-day assay (repeatability), inter-day or intermediate assay, and reproducibility. Repeatability is evaluated for operational conditions (same analyst, same equipment, among others experimental conditions) at the smaller time interval as possible. At the same time, intermediate precision is obtained at distinct operating conditions (different analyst, equipment, or day). These assays produce numerical values that express the random error or the dispersion's degree for a group of measurements. They will be appraised using calculations of standard deviation (SD), variance, or the coefficient of variation (RSD).

In its turn, accuracy is described as the closeness of agreement between a test result and a reference value accepted as the true value. There are four primary forms to evaluated estimate accuracy: *(i)* comparing an experimental result to certified reference materials (CRMs) values; *(ii)* comparing the proposed method to the reference method; *(iii)* executing a recovery test using sample matrix; and *(iv)* collaborative studies (ARAUJO, 2009; INMETRO, 2016).

The validation parameter that reveals the influence of minimal variations of factors from the method is called robustness. For chromatographic purposes, it is common to evaluate robustness for flow rate, mobile phase composition, column temperature, different brands of reagents, distinct lots of similar phase columns, *etc*. These variables should be changed deliberately to identify the experimental factors that, even when varied, maintain appropriate precision and accuracy. Independent from the approval or rejection of some selected factor in robustness assessment, it is possible to carry on with the validation process; thus, many validation guides do not determine the evaluation of this parameter (PERIS-VICENTE, ESTEVE-ROMERO, CARDA-BROCH, 2015; MARSON *et al*., 2020).

3.2 Aim

In this chapter, we aimed to evaluate the presence or absence of PCB in ultra-high temperature (UHT) whole-milk samples from the Brazilian state of São Paulo, the most populous and industrialized amongst the Brazilian states.

3.2.1 Specific objectives

- Validation of miniaturized QuEChERS sample preparation method for PCB extraction from UHT whole-milk samples followed by gas chromatography with triple quadrupole mass spectrometer detection (GC-MS/MS) analysis.
- Assessment of PCBs contamination in UHT whole-milk samples from São Paulo State.

3.3 Experimental

3.3.1 Reagents, solutions, and equipment

Individual PCBs congeners standards (congeners 28, 52, 101, 118, 138, 153, 156, 170, 180) were purchased from AccuStandard (New Haven, USA). Standard solutions of congeners were prepared at isooctane (HPLC grade). Dodecachlorobiphenyl (PCB 209) was used as an internal standard (ISTD) in a 0.5 μg mL^{-1} solution obtained from Cerilliant (Round Rock, USA). Anhydrous magnesium sulfate (≥99.5%), sodium chloride (≥99.5%), trisodium citrate dihydrate (≥99.0%), disodium hydrogen citrate sesquihydrate (≥99.0%), SupelClean^{TM} PSA (primary-secondary amine) silica, cyclohexane (≥99.5%) were acquired from Sigma Aldrich (Saint Louis, USA). Acetonitrile (HPLC grade) was purchased from da Honeywell (Raunheim, Germany), and isooctane (HPLC grade) was acquired from J.T. Baker (Darmstadt, Germany).

The centrifuge used was a Heraeus Megafuge 40R model from Thermo Fisher Scientific (Langenselbold, Germany), and the vortex mixer S0200 model was purchased from Labnet International Inc. (Edison, USA).

3.3.2 Instrumentation

It was utilized the same instrumental conditions for GC-MS/MS analysis as described in section 2.3.2.

3.3.3 Analytical validation procedure

Samples were prepared using a 1.7 mL of UHT whole milk spiked with PCBs standard solution to provide the desired concentration level of linear range in the sample, also being

added ISTD solution. It was added 1 mL of ultrapure water, 2 mL of acetonitrile, and 1.3 g of a mixture of MSQ1, followed by 30 s of vortex agitation and centrifugation (5 min at 2580 RCF). Two re-extraction steps of 1 mL of MeCN increments were carried out, with a homogenization-centrifugation after each addition. A 4-mL of the supernatant was transferred to another tube containing 280 mg of MSQ2 (100 mg of PSA + 100 mg $MgSO_4$), and one more agitation-centrifugation stage was carried out. Then 2.5 mL of extract was pipetted, dried under slight N_2 flow, resuspended into 500 µL of cyclohexane, and filtered to an injection vial.

The method validation was performed according to DOQ-CGRE-008 from August 08, 2016, from the National Institute of Metrology, Quality and Technology (INMETRO, from Brazilian Portuguese "*Instituto Nacional de Metrologia, Qualidade e Tecnologia*") and SANTE/11813/2017 guidelines (EUROPEAN COMMISSION, 2017).

Selectivity was the first validation parameter appraised to verify the presence of interference compounds. Twenty blank samples of UHT whole-milk from distinct origins were prepared and analyzed by GC-MS/MS, and then the average peak area of blank samples was compared to the average peak area of six samples spiked with PCBs standard solution at the lowest analytical range level. We also evaluated the matrix effect as a complementary study of selectivity by comparing between an analytical curve spiked with PCBs and a blank extracted curve with posterior fortification at the end of extraction procedure.

A preliminary linearity study was conducted using a linear range from 2.50 to 100.00 ng mL^{-1}, in order to contemplate European MRL for PCBs in milk and dairy products. For quantitation of real samples, a narrow range (0.05 to 2.50 ng mL^{-1}) was used to decrease deviations and contemplate the concentrations samples better. The linearity of each PCB congener dynamic range was appraised by preparing six replicates for each concentration level and blank sample. The obtained data of the linearity study was evaluated through linear fitting by using least-squares method.

Limit of quantification (LOQ) for all PCB congeners was taken as the first calibration level with suitable selectivity, precision, and accuracy. Limit of detection (LOD) was achieved as three times blank standard deviation *(n=6)* divided by the slope of analytical curve of each analyte.

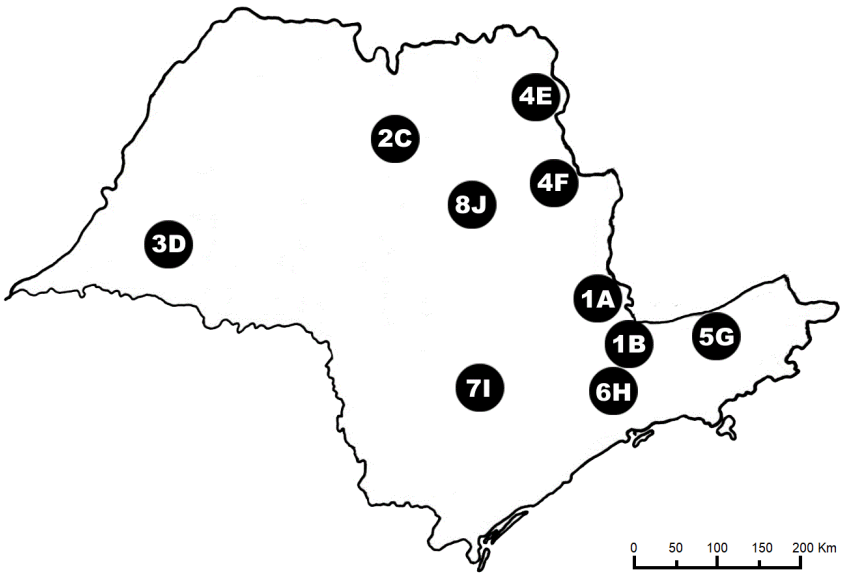
Intraday precision (repeatability) was evaluated by considering the RSD of six replicates prepared and analyzed in the same day of UHT whole-milk sample spiked with PCBs standard at three quality control (QC) levels: QC at low concentration (QCL = 0.10 ng mL^{-1}); QC at the medium concentration (QCM = 0.50 ng mL^{-1}); and QC at high concentration (QCH = 2.00 ng mL^{-1}). Intermediate precision was assessed in three days of sample preparation at QCL, QCM,

and QCA, with a minimal interval of 24 hours between them. The acceptance criteria applied were RSD lower than 20% and recovery rates varying from 70 to 120% for replicate analysis (*n = 6*, for repeatability). Accuracy appraisal was performed through recovery assay at the same analytical concentrations of QC levels, being accepted recoveries from 70 to 120% for the repeated analysis (INMETRO, 2016).

3.3.4 Sample collection and analysis

Ten local dairy brands of São Paulo state were selected to represent eight dairy farming regions. Samples of UHT whole milk were purchased during the first semester of 2019 in markets all over the state to provide a representative sample. The map of Figure 11 represents where each dairy brand performs its milk processing through São Paulo state. Each brand was collected 3 sample units (box of UHT whole milk) from 3 different lot numbers (except for one brand, which had just one lot collected), totalizing *n = 81* samples. All samples were analyzed before the expiration date to guarantee consumption conditions as close as possible to real-life consumption.

Figure 11 - Sampling sites of UHT whole-milk samples from São Paulo state, Brazil



Legend: Dairy farming are represented in this figure by the numbers - (1) Campinas, (2) São José do Rio Preto, (3) Presidente Prudente, (4) Ribeirão Preto, (5) Vale do Paraíba, (6) São Paulo, (7) Itapetininga, and (8) Araraquara. Each letter in the map indicates one local milk brand collected.
Source: Letícia A. Marques (2021).

3.3.5 Data analysis

It was used ActionStat™ Pharma software (Estatcamp, São Carlos – SP, Brazil) and Microsoft™ Excel 2010 (Microsoft Corporation, Redmond - WA, USA) to treat validation data

and to tabulate raw data, respectively.

3.4 Results and discussion

3.4.1 Validation of the analytical method

A preliminary study of linearity (ranging from 2.5 to 100.00 ng mL^{-1}) indicated good linear parameters performance. However, when we carried out some quantification test of real UHT whole-milk samples, we notice a necessity of linear range adjustment to contemplate best the probable concentrations of PCBs in the samples. Therefore, a matrix effect study was appraised to this wide linear range by three assays: intercept equality test, parallelism test, and coincidence test. Data of the matrix effect study are presented in Table A5 (Appendix A). We observed no significant values of *p* for both parallelism and coincidence tests for all analytes, which indicates that analytical curves (in solvent or matrix) do not either parallel neither have the same slope coefficient values. In this way, it was verified that both the analytical curves in solvent and milk are different. Afterward, from a critical analytical standpoint, the proposed method must always be performed with matrix curves construction, whether using wide or narrow linear ranges.

Table 10 is also shown selectivity and linearity results of the narrow range assessed (0.05 – 2.50 ng mL^{-1}). At first, we observed that the method of PCBs extraction using a miniaturized QuEChERS followed by GC-MS/MS analysis is selective to all analytes at 0.05 ng mL^{-1}, except for PCB 28 is selective at 0.10 ng mL^{-1}. This is asserted once the area ratio of blank replicates and the first concentration level were below 30%.

For congeners 118, 156, 180, and 170, the obtained coefficients of determination were $R^2 > 0.98$, while linear correlation coefficients were $R > 0.990$, indicating that more than 99% of data are well explained for the linear model. The rest of the congeners linear models reached R^2 and R values higher than 0.99, showing a strong linear correlation (greater than 99% of the obtained information is well described by the linear model). All regressions were significant since F-test values were much higher than 100 and *p*-value lower than the significance level (0.05). Regarding the homoscedasticity evaluation, we observed that none of PCBs linear ranges presented homoscedastic comportment according to the Brown-Forsyte test (*p-value* > 0.05), a weighted least square was applied. Linear regressions models obtained for PCBs congeners showed a heteroscedastic behavior (*p-value* < 0.05 at Brown-Forsyte test). It indicates that residues are more diffuse along with the linear range, and confidence intervals are adjusted through lower values (MARSON *et al.*, 2020; SOUZA, JUNQUEIRA, 2005).

Besides, it is common to observe heteroscedasticity for datasets of large ranges. The relevance of this observation is performing the proper linear regression fitting. Once OLS presumes that all residues of a population have homogeneity of variance along all linear range, this type of regression is not efficient or proper to use it to data treatment of heteroscedastic results; soon this implicates that the WLS is the suitable linear regression type to fit data of PCB congeners.

Precision (intraday and intermediate) and accuracy results are presented in Table 11. The obtained RSD values for the intra-day and intermediate precision essays were lower than 20%, demonstrating a suitable precision for PCBs analysis. This method could also be considered suitable accuracy once recovery rates ranged from 95.81 to 112.83% to all congeners in the QC levels evaluated.

3.4.2 Analysis of UHT-whole milk samples

Using the proposed and validated method, analysis of 81 UHT whole-milk samples from the São Paulo state dairy basin, collected in the first semester of 2019, did not show quantification levels of PCBs, *i.e.*, all samples were below LOQ values. Chromatograms of a 1 ng mL^{-1} standard sample and a real milk sample are presented in Figure 12. The standard sample total ion chromatogram (TIC) presented, visually, a good separation of the monitored analytes. To better observe quantification and confirmation of this analysis, PCBs congeners 28 and 170 were chosen to be highlighted. We see that individual quantitation transitions presented resolved and thin peaks; confirmation transitions chromatograms are observed overlapped at the same RT as quantitation transition. It is also noticed the expected ion ratios (as showed in Table 2) for each confirmation transition. All these criteria were detected for each PCB congener in each injection. On the other hand, the TIC chromatogram of the real sample almost does not present analyte signals, while it is easy to observe ISTD signal. The two confirmation transitions set for PCBs 52 or 170 were not found or not presented the expected ion ratio. When these specified criteria are not achieved, the MassHunter software highlight in orange that analyte for easier identification of analyte signal absence. This visual evaluation was essential to confirm that there was no quantification of PCBs for the 81 samples analyzed.

Our PCBs assessment of the São Paulo state UHT whole-milk samples corroborates with Schröder, Pinhel, and Mendonça (2016), in which organochlorine compounds (OCPs and PCBs) were evaluated on animal fat tissues. According to them, contamination levels of PCBs in Brazil are not exceeding the limiting levels in the latest years, which implies reducing the sample numbers to be analyzed as part of the National Plan for Control of Residues and Contaminants (PNCRC) instituted by MAPA.

Table 10 - Selectivity, linearity performance parameters, LOD, and LOQ of the proposed method

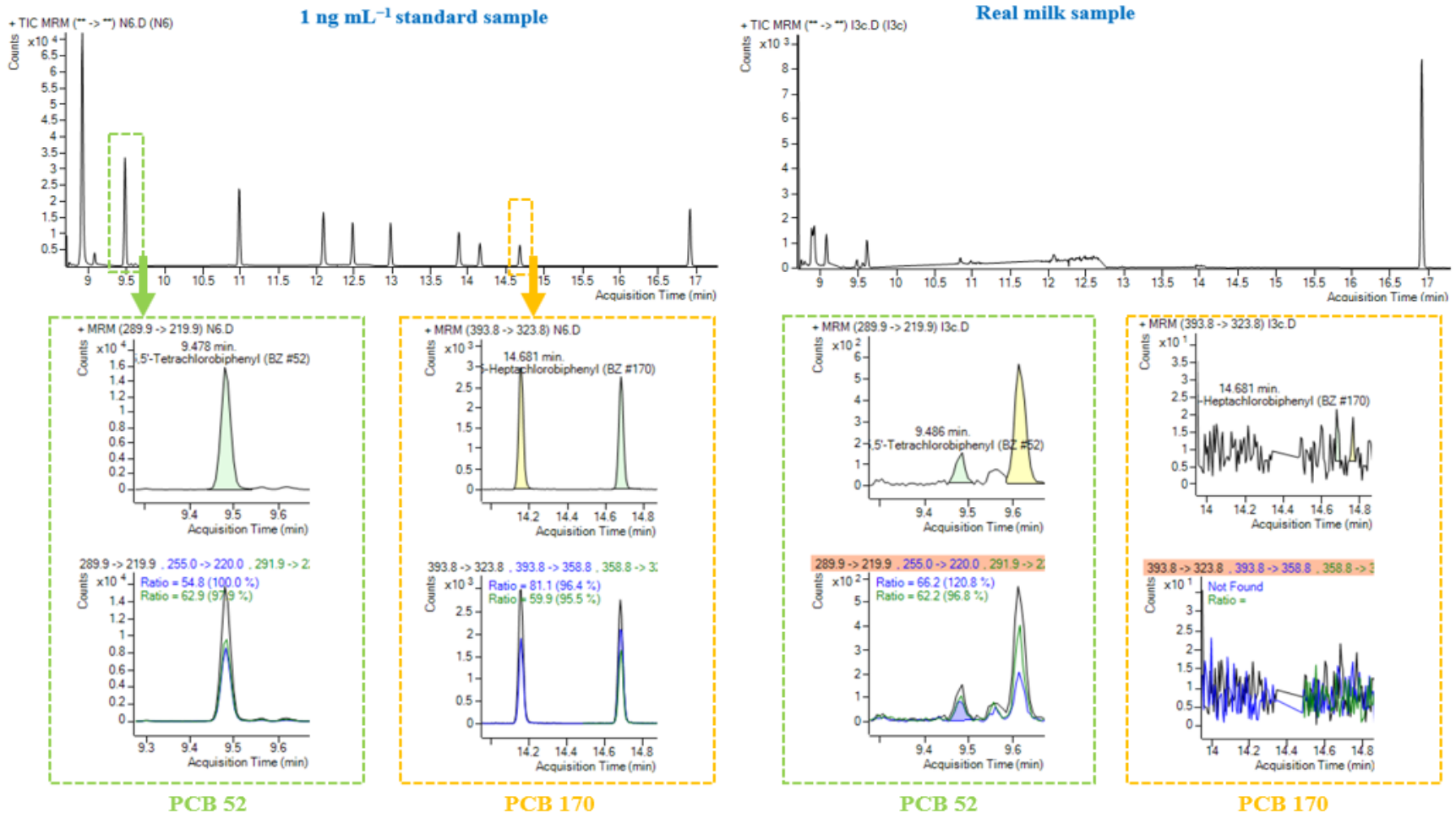
Analyte	***Selectivity***	**Linearity parameters**								***LOD***	***LOQ***
	Ratio%	*Regression type/weight*	*Linear range (ng mL^{-1})*	*Cal. levels*	*R²*	*R*	*F*	*p*	*Equation*	*(ng mL^{-1})*	*(ng mL^{-1})*
PCB 28	25%	WLS, 1/x	0.10-2.50	6	0.9933	0.9966	5011.61	1.66E-38	y = 3622.13 + 58309.72 x	0.008	0.100
PCB 52	18%	WLS, 1/x	0.05-2.50	7	0.9914	0.9957	4585.50	6.89E-43	y = 604.53 + 25192.11 x	0.005	0.050
PCB 101	10%	WLS, 1/x	0.05-2.50	7	0.9915	0.9957	4647.71	5.27E-43	y = 487.98 + 20186.90 x	0.003	0.050
PCB 118	10%	WLS, 1/x	0.05-2.50	7	0.9899	0.9949	3915.62	1.57E-41	y = 172.70 + 13612.44 x	0.003	0.050
PCB 153	6%	WLS, 1/x	0.05-2.50	7	0.9929	0.9964	5593.72	1.33E-44	y = 109.49 + 10112.70 x	0.005	0.050
PCB 138	7%	WLS, 1/x	0.05-2.50	7	0.9928	0.9964	5548.61	1.57E-44	y = 108.25 + 8653.73 x	0.005	0.050
PCB 156	8%	WLS, 1/x	0.05-2.50	7	0.9886	0.9943	3455.92	1.86E-40	y = 77.43 + 7934.72 x	0.005	0.050
PCB 180	7%	WLS, 1/x	0.05-2.50	7	0.9875	0.9937	3167.03	1.05E-39	y = 61.04 + 5865.93 x	0.008	0.050
PCB 170	8%	WLS, 1/x	0.05-2.50	7	0.9896	0.9948	3794.27	2.94E-41	y = 34.99 + 4698.40 x	0.010	0.050

Table 11 - Precision and accuracy results of the proposed method

Analyte	***Intraday precision***			***Intermediate precision and accuracy***					
	QCL	*QCM*	*CQH*	*QCL*		*QCM*		*QCH*	
	RSD (%)	RSD (%)	RSD (%)	RSD%	Rec%	RSD%	Rec%	RSD%	Rec%
PCB 28	10.39%	9.02%	2.12%	11.11%	112.83%	6.48%	106.93%	7.49%	98.27%
PCB 52	2.74%	9.99%	2.68%	8.62%	103.62%	7.60%	105.68%	7.82%	99.34%
PCB 101	2.04%	9.14%	3.23%	7.99%	102.10%	7.12%	106.57%	7.99%	98.90%
PCB 118	6.16%	9.39%	2.76%	10.69%	98.11%	8.16%	105.04%	9.54%	97.38%
PCB 153	6.22%	7.46%	2.83%	7.38%	97.54%	6.04%	104.69%	8.26%	97.07%
PCB 138	3.24%	8.51%	3.67%	9.46%	99.98%	6.57%	106.66%	8.75%	97.65%
PCB 156	3.22%	7.45%	3.24%	8.61%	98.56%	6.59%	105.61%	9.21%	96.24%
PCB 170	5.66%	7.82%	2.97%	7.66%	97.68%	6.47%	105.16%	9.18%	95.81%
PCB 180	5.91%	7.00%	3.32%	9.87%	97.57%	6.82%	107.09%	9.53%	96.31%

Legend: QCL – quality control at low concentration (0.10 ng mL^{-1}); QCM – quality control at the medium concentration (0.50 ng mL^{-1}); QCH – quality control at high concentration (2.00 ng mL^{-1}); Rec – recovery.

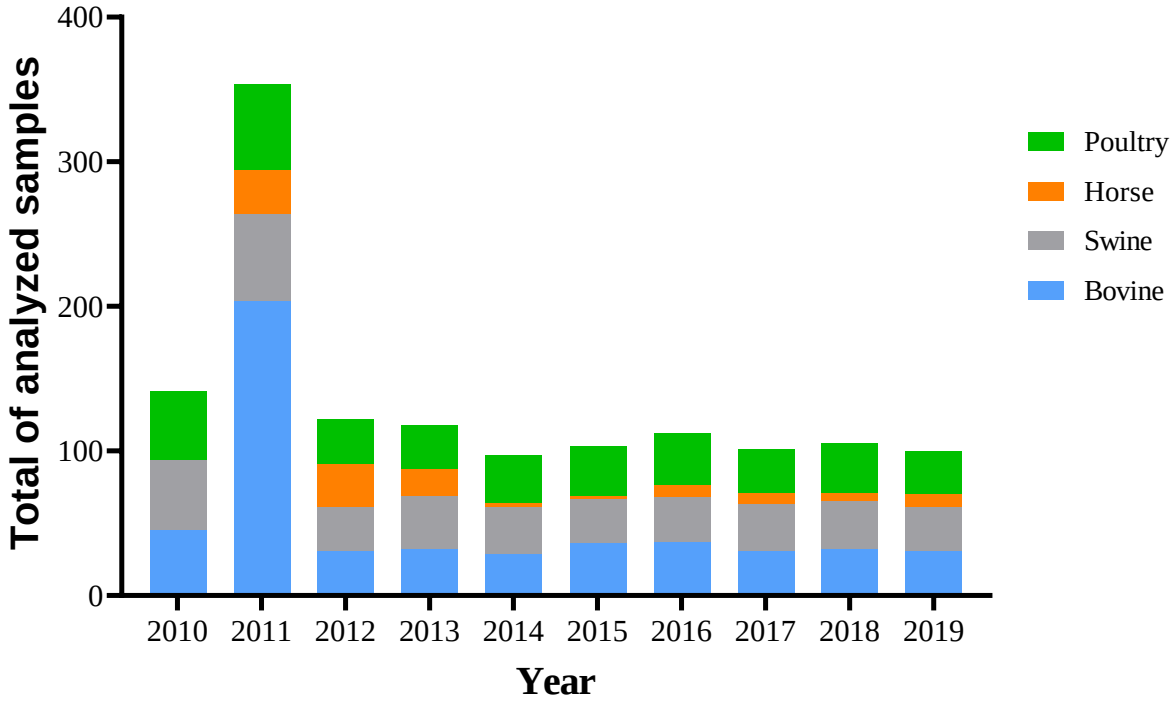
Figure 12 – Comparison TIC and MRM chromatograms for standard samples and real samples



Legend: Identified peaks are filled with green color. Highlighted transitions (in orange) indicate that no confirmation transitions were found or did not present the expected ion ratio.
Source: Letícia A. Marques (2021).

A data collection of PNCRC results for organochlorine compounds in animal origin matrices does not show nonconformity for any 1353 samples analyzed from the 2010 to 2019 period (see Figure 13). As cited in Schröeder, Pinhel, and Mendonça study (2016), the Brazilian strategy for monitoring residues of 18 organochlorine compounds[1] is currently restricted to their analysis of adipose tissue of cattle, poultry, horses, and swine. Another organochlorine group[2] has been monitored in fish and honey; in this same period, a total of 531 samples were assessed for this second organochlorine group, also presenting no exceedances of MRL established. The Brazilian legislation also demands monitoring dioxin-like compounds, including PCDDs, PCDFs, and the DL-PCBs congeners, all of them presenting a correlated WHO-TEF value previously shown in Table 1 (Chapter 1).

Figure 13 - Latest ten years survey of organochlorine's analysis in main animal matrices foreseen PNCRC



Source: Letícia A. Marques (2021), made from PNCRC data reports available in the reference (BRASIL, 2020).

[1] Includes at least the following compounds: aldrin, α-HCH, HCB, dieldrin, dodecachlor, heptachlor, heptachlor epoxide, cis-chlordane, trans-chlordane, pp'-DDT, pp'- DDE, op'-DDT, pp'-DDD, PCB 101, PCB 118, PCB 138, PCB 153, PCB 180. This group had incorporated lindane and o,p'-DDE to the monitored compounds through years of the reported period

[2] This group of compounds varied in number and type of molecules from one year of monitoring to the next and may include other compounds, such as vinclozolin, tetradifon, etc. In some years, the monitoring of these two matrices was not performed.

Currently, it is not established a value of MRL for the sum of NDL-PCBs indicators (congeners 28, 52, 101, 118, 138, 153, and 180) in Brazil; even though an MRL of 40 ng g^{-1} fat was took by European Community according to Regulation (CE) Nº 1881/2006, as amended by Regulation (CE) Nº 1259/2011 (EUROPEAN COMMISSION, 2011).

Our proposed and validated methodology is an option for PNCRC to monitor six PCBs indicator congeners and three other congeners (PCBs 118, 156, and 170) from bovine milk samples.

3.4 Conclusions

The proposed miniaturized QuEChERS extraction for PCBs from UHT whole-milk samples followed by GC-MS/MS determination could be successfully validated, presenting LOQs from 0.05 to 0.10 ng mL^{-1}. Furthermore, it was noticed that the method is appropriate to perform real samples analysis. Moreover, miniaturized QuEChERS followed by GC-MS/MS analysis might be recommended to monitor NDL-PCBs indicators congeners in milk when the demand exists in Brazil.

Sample collection of UHT whole-milk samples all over São Paulo state was considerately representative. The assessment of these samples indicates no PCBs residues regarding the linear range and LOQ of the proposed method. These results are aligned with the latest ten years of PNCRC.

CHAPTER 4 – Development of a low-cost microfluidic platform with C^4D detection for an organochlorine compound as a semi-quantitative aptasensor

4.1 Introduction

4.1 Fabrication of microfluidic devices

Essentially, microfluidic devices are platforms obtained from a well-established fabrication technology capable of integrating constituent parts, ideally creating interconnected pathways adaptable to several different tests. This platform affords to execute an essential unit operation of fluids, such as transporting, metering, mixing, valving, separation, or concentration. As previously mentioned, micro-total analysis systems (μTAS) or "lab-on-a-chip" (LOC) are those devices capable of carrying out complete lab procedures inside its platform. They are major known for their portability, great sensibility, cheap fabrication costs, easy automation, wearability, *etc*. There are also other microfluidic platforms, such as micro-dosage systems and micro-processes engineering (HAEBERLE & ZENGERLE, 2007; COLTRO *et al*., 2014; MARK *et al*., 2010).

Microchips can be created over a substrate in which microscopic channels or chambers will be manufactured. Materials as ceramics (*e.g.*, glass), polymers (like PDMS, polycarbonate, poly (methyl methacrylate) – PMMA, *etc*.), semiconductors (silicon, elastomers), hydrogels, and even paper can be used as microfluidic devices substrates (HAEBERLE & ZENGERLE, 2007; REN, ZHOU, WU, 2013).

After choosing a substrate material, microchips can be manufactured at the selected design through some processes such as hot embossing, lithography, laser photoablation, injection molding, imprinting (wax printing, inkjet printing), and more recently, three-dimensional (3D) printing. For instance, conventional photolithography is one of the most used techniques to create microchannels or electrodes. A sensitive material to ultraviolet (UV) radiation, known as photoresist, is used to create geometric patterns over the surface of substrates; after UV exposure, the desired pattern is transferred to the substrate, enabling the production of microchannels or metallic electrodes through corrosion or thermic processes. Despite great reproducibility, photolithography is expensive, requiring specialized facilities and professionals to perform it. Furthermore, to finalize the production of microfluidic devices, they must be sealed to guarantee system enclosure (COLTRO, 2008; COLTRO *et al*., 2014; FIORINI & CHIU, 2018).

However, microfluidic platform construction is not so trivial as it seems to be. Either conventional substrates or microchannel patterning used to be expensive, requiring specialized equipment, high-priced consumables, and facilities, as a clean room, for microdevices fabrication. These factors certainly hamper the spread and development of microfluidics

research in developing countries, where universities or research institutes have restricted funding to invest in such an expensive structure. Then, the fabrication of low-cost microfluidic devices became a growing research line, aiming to establish easier prototype manufacturing, requiring facilities investments, minimizing developing time, and of course, reducing the cost production (COLTRO *et al*., 2014; KRATZ *et al*., 2019; SHARMA *et al*., 2011).

Several studies have reported using double-sided adhesive tapes, known as pressure-sensitive adhesive, and polyester sheets (laser printer transparency films) to fabricate microchannels in an easy, fast, and low-cost compared to classic microfabrication methods. Besides the economic aspect, tapes of pressure-sensitive biomedical adhesive are an attractive option to bond or assemble on various substrates, with advantages such as: *(i)* quick production, allowing industrial-scale production; *(ii)* biocompatibility, which is crucial to the biological recognizing system of LOC platforms and organ-on-a-chip devices (HUI LING *et al*., 2021; KRATZ *et al*., 2019; SHARMA *et al*., 2011).

Finally, detectors types employed in microfluidics application vary since optical methods (as fluorescence, colorimetry), mass spectrometry, electrochemical methods, and other miscellaneous methods, as surface-enhanced Raman spectroscopy (SERS), luminescence, *etc*. (COLTRO *et al.*, 2014; MAZAAFRIANTO *et al*., 2018).

4.2 Electrochemical detection in microfluidics devices

Electrochemical systems had been seen as a natural option to signal transduction in microdevices because they possess high compatibility with microfabrication processes and advanced micromachining, besides their high sensitivity (comparable to fluorescence) and an affordable production cost. Moreover, it is simple to integrate into microdevices, being ideal for point-of-care (POC) devices. They also have been gaining ground in capillary zone electrophoresis and ultra-fast flow-injection analysis. These electrochemical techniques assess the oxidation-reduction activity of an analyte in solution, *i.e.,* electron transfer involved in chemical reactions (WANG, 2002; RACKUS, SHAMSI, WHEELER, 2015).

It is essential to observe the materials of electrodes and substrates for microfluidic devices. The most common substrates to fabricate electrodes are glass and silicon, whist carbon, platinum, and gold are the most used to obtain electrodes. It is possible to manufacture electrodes using thin-film or sputtering deposition, conductive pastes, or inks, and performing pencil drawing (WANG, 2002; RACKUS, SHAMSI, WHEELER, 2015).

Regarding applications, the most used electrochemical techniques are amperometry and voltammetry for catalytic mode measures, and electrochemical impedance spectroscopy (EIS)

is more applicable to affinity biosensing. However, other well-known electrochemical techniques, *e.g.*, conductivity or potentiometry, have also been applied to microchip production. Therefore, the following section will introduce and review a contactless conductivity-based technique very suitable for microfabrication purposes.

4.2.1 Capacitively coupled contactless conductivity detection

Capacitively coupled contactless conductivity (C^4D) is an electrochemical technique based on the system's total admittance, *i.e.*, the inverse of impedance solution. Opposite to contact conductivity, electrolyte solution does not enter in direct contact with electrodes of a C^4D system. In microfluidic devices, the electrode-insulator-solution conjoint presents a similar behavior to a parallel plate capacitor. Also called contactless conductivity detection (CCD) or oscillometric detection, C^4D responses for any ion in solution, and it can be considered a universal detector for electro driven separation techniques, as capillary electrophoresis (DA SILVA & DO LAGO, 1998; FRANCISCO & DO LAGO, 2009; COLTRO *et al*., 2012).

The first application of a C^4D system dated to the 1980s when Gas and collaborators published an isotacophoretic determination of organic and inorganic ions; nevertheless, C^4D remained underutilized for many years, until two decades later, when Zeeman *et al*. and da Silva and do Lago independently proposed, in 1998, innovation in C^4D electrodes arrangement to the axial configuration. Since then, the number of publications using C^4D detectors to capillary electrophoresis and microfluidics determination increased substantially, placing this electrochemical technique between the consolidated ones and commercially available. Since then, the interest for C^4D detectors to capillary electrophoresis and microfluidics applications have been increased substantially, placing C^4D into a highlighted place between consolidated electrochemical techniques (COLTRO, 2008; COLTRO *et al*., 2012; DA SILVA & DO LAGO, 1998; ELBASHIR *et al*., 2020; FRANCISCO & DO LAGO, 2009; ZEEMAN *et al*.; 1998).

A basic C^4D system is constituted of electrodes physically isolated from solution contact through a dielectric layer; then, the faradaic current is absent in this system. A usual C^4D microcell into a microfluidic device is presented in Figure 14 (A), constituted of *(i)* one capacitor (C_1 and C_2) for each electrode; *(ii)* a resistor formed by the electrolytic solution inside the microchannel or capillary, resulted from the current flow difficulty to flow through the solution migration (also called solution resistance); *(iii)* and the stray capacitance (C_s), which refers to the capacitive coupling between the electrodes. The resultant capacitance of C_1 and C_2 is known as the capacitance of the electrode geometry and wall thickness (C_w), described by Equation 16 and Figure 14(B):

$$C_w = \frac{C_1 C_2}{(C_1 + C_2)}$$ *Equation 16*

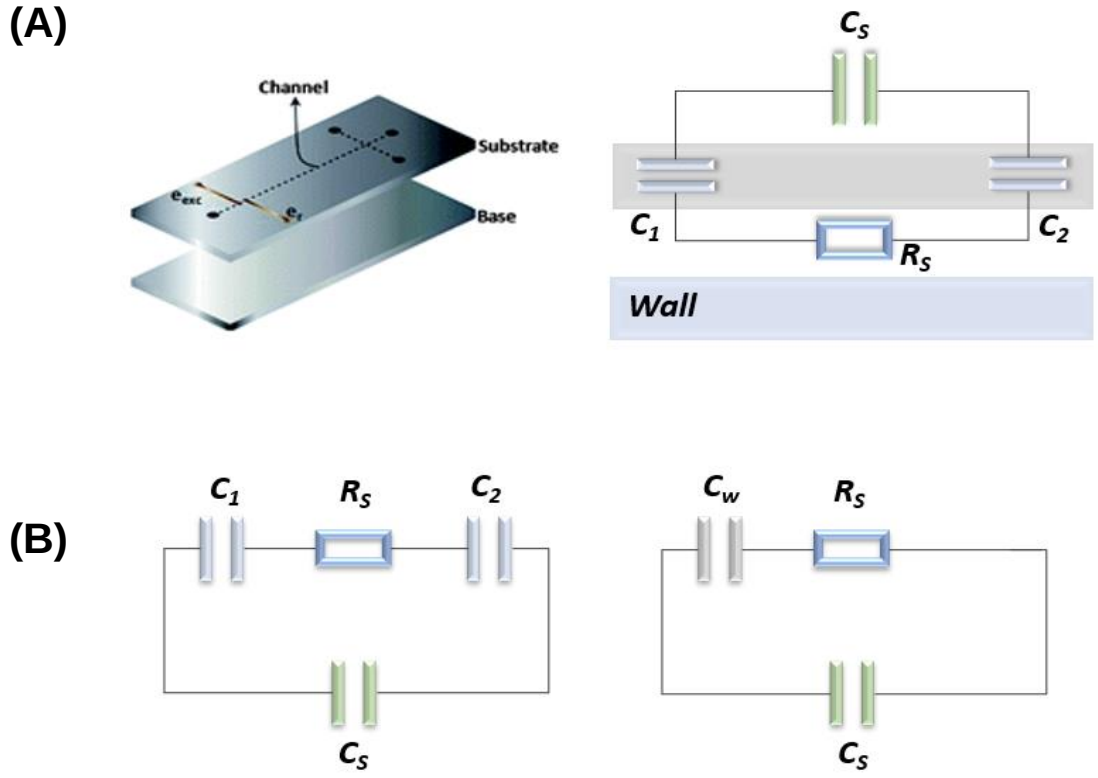
Thus, it is essential to maintain wall capacitance at the electrode's surroundings as high as possible, while stray capacitance should be kept as low as possible (COLTRO *et al*., 2012).

Moving forward into definitions, a solution's conductivity is the product of the concentration of ionic species (c_i) and their molar conductivities (λ_i). Conductance (G) is described as a function of the conductivity (κ) and some electrochemical cell parameters, as given by Equation 17:

$$G = \kappa \frac{A}{l}$$ *Equation 17*

Where G is conductance (S), A is electrode area (m²), l is the electrodes distance (m), κ is the solution conductivity ($\kappa = \sum c_i \lambda_i$, expressed in S m^{-1}) (KUBÁŇ & HAUSER, 2009; GAMAT, FOTUOHI, TALEBPOUR, 2017).

Figure 14 – Typical C^4D system configuration in a microfluidic device (A) and equivalent circuit in terms of capacitances (B)



Source: adapted from COLTRO *et al*., 2012; COLTRO, 2008.

In this electrochemical system, there is the application of an alternating current (AC) excitation signal in the first electrode, promoting a capacitive coupling of the electrolyte, which will provide, in its turn, a current flow, measurable by the second electrode in the cell. In other words, it occurs a formation of a capacitor between the electrodes of the C^4D system and the electrolytic solution. Then, the obtained current is amplified by an electronic circuit (BRITO-NETO *et al.*, 2005; COLTRO, 2008; COLTRO *et al.*, 2012; GAMAT, FOTUOHI, TALEBPOUR, 2017).

Towards to describe C^4D measurements using mathematical expressions, it is essential to elucidate some points. First, conductance signals are proportional to increases in analytical concentration, but resistance signals are not. The second aspect, once a microchannel is filled with an electrolyte solution, the admittance of the system is a function of the conductance in the region between the two electrodes. There is also a phase shift between the input AC voltage and the generated current, which is usually ignored (BRITO-NETO *et al.*, 2005). Considering the stated circumstances, the modulus of measured admittance $|Y|$ is given by Equation 18:

$$Y = \frac{1}{\sqrt{\left(\frac{K}{\kappa}\right)^2 + \frac{1}{4\pi^2 f^2 {C_w}^2}}} \qquad \textit{Equation 18}$$

Where Y is admittance, K is the cell constant, κ is the solution conductivity, f is the frequency of operation, and C_w is the wall capacitance.

The AC signal is a sinusoidal wave of constant amplitude, capable of promoting dielectric layer polarization. Also, the usage of AC signal reduces electrolysis effects on the conductivity determinations (BRITO-NETO *et al.*, 2005; FRANCISCO & DO LAGO, 2009).

There is no doubt about the importance of frequency and amplitude of the signal for C^4D ideal performance, the reason why many researchers have been studying these experimental features to optimize the signal. Observed capacitances of C^4D electrodes are lower than double-layer capacitance without insulation. Due to that, higher-frequency signals (of several kHz) are employed in C^4D, being 300 kHz a typical operating frequency. Operation frequency depends mainly on cell geometry and also on the electronics of the detector (BRITO-NETO *et al.*, 2005; FRANCISCO & DO LAGO, 2009; KUBÁŇ & HAUSER, 2008; KUBÁŇ & HAUSER, 2009).

Despite being inherent to the C^4D system, stray capacitance is undesirable because it minimizes sensibility; so, it is suggested to resort to ground electrode usage or optimizing frequency to avoid as much as possible the stray capacitance. It can also perturb peak height, background signal rise, and signal-noise deterioration (COLTRO, 2008; COLTRO *et al*., 2012).

There are advantages of C^4D detectors, such as less requirement of electrode cleaning, avoid of bubble formation from electrolysis process, avoid of electrode fouling, absence of electrode contamination, minimizes lack of reproducibility of electrodes surface, which results in better performance, and lower cost of electrodes fabrication. An additional benefit is that C^4D has good performance on biomolecules detection when coupled to capillary electrophoresis, and for the construction of C^4D-based biosensors. (BRITO-NETO *et al*., 2005; COLTRO, 2008; COLTRO *et al*., 2012; ELBASHIR *et al*., 2020).

The main disadvantage of C^4D is its lower sensitivity compared to other electrochemical detection modes, as amperometry or even direct conductivity. However, there are three main possibilities to improve C^4D sensitivity: *(i)* increasing the electrode detection area; *(ii)* decreasing the dielectric thickness; and *(iii)* reducing stray capacitance.

4.3 Biosensors

Biosensors are devices on which specific biochemical reactions act to molecular recognizing (receptor) and, in general, perform analytical signal transduction through optical, electrical, or thermic properties. They are used for biological or non-biological sample monitoring (IUPAC, 2006). Studies in the biosensors field look for innovations related to transduction principles and surface functionalization of the substrates, allowing more versatility and biosensors' applicability. Also, it enables to improve the number of target molecules to be determined and increase sensitivity and selectivity.

Several biorecognition systems can create a transducer surface layer, such as DNA/RNA, antibody-antigen, aptamer-ligand, or even cells. Affinity biosensors are a class of biosensors. A permanent or semi-permanent binding between analyte and bioreceptor molecules will promote a physicochemical property as current flow, heat transfer, mass changing, refractive index alteration, *etc.*

Lately, aptamers have been spread as the main element of the biorecognition system of electrochemical-based microdevices called aptasensors (MING *et al.*, 2020). Aptamers are defined as oligonucleotides (single-stranded DNA or RNA) or peptides molecules from synthetic origin capable of specific binding on target molecules through Van der Waals forces, hydrogen bonds, or electrostatic interactions. They behave similarly to antibodies, with the

advantages of being more stable and less expensive; moreover, chemical synthesis favor modifications on oligonucleotides, as a change in the extremities of a sequence or even addition of electroactive indicators (MAZAAFRIANTO *et al*., 2018; RACKUS, SHAMSI, WHEELER, 2015). Also, aptamers can be regenerated without losses in sensitivity or selectivity, making them an interesting alternative to antibody-antigen immunosensors.

4.2 Aim

This chapter aimed to construct a low-cost C^4D microfluidic device with selective DDT detection based on aptamer-ligand biorecognition.

4.2.1 Specific objectives

- Develop a microdevice with C^4D detection using low-cost materials and fabrication techniques.
- Evaluate functionalization of the working electrode in two different biorecognition systems: antibody-antigen and aptamer-ligand.
- Perform immobilization of the organochlorine DDT through a specific interaction with respective synthesized aptamer.

4.3 Experimental

4.3.1 Reagents, solutions, and equipment

Glass coverslip of dimensions 24 × 60 mm and average thickness of 0,13 – 0,16 mm (model K5-2460) were acquired from Olen. Polyester sheet (A4 size -210 × 297 mm, 100 µm thickness) was obtained from Filipaper (Rio de Janeiro, RJ, Brazil). Pressure-sensitive adhesive ARcare™ 90106 were kindly offered by Adhesives Research Inc. (Glen Rock, EUA). The conductive silver paste was acquired from Spi Supplies (West Chester, PA, USA). Intravenous infusion microtubes (BD Asepto® 21 GA), pipette tips of 10 µL (Eppendorf) were used to produce inlets and outlet connections. Epoxy glue Araldite™ was purchased from Tekbond (Brazil).

Isopropyl alcohol, acetone, and ethanol were purchased from Neon (Suzano, São Paulo – Brazil). Sulfuric acid, hydrogen peroxide, and potassium hydrogen phosphate were acquired from Synth (Diadema, São Paulo - Brazil). Ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate was obtained from Qhemis (Brazil). Disodium hydrogen phosphate was produced

by Mallinckrodt. Tris-base (>99.9%), sodium chloride (>99.5%), bovine serum albumin (BSA) in lyophilized powder, glutaraldehyde solution at 50% in water, and (3-aminopropyl)triethoxysilane (APTES) (99%) were purchased from Sigma Aldrich. Anti-BSA at 2 mg mL^{-1} was purchased from Thermo Scientific. DDT was obtained from Supelco. The aptamer synthesis was performed by Exxtend Biotecnologia (Paulínia, São Paulo – Brazil).

4.3.2 Fabrication of the microfluidic platform

Initially, metallic electrodes were deposited over a glass layer for the design shown in Figure 15 (A). Next, AutoCAD drawings created adhesive paper masks, then cut in a CO_2 laser cut (Laser600, Combat Laser Kawaii), followed by the mask fixed to the glass substrate surface.

The metallic deposition was carried out by thermal evaporation using a Balzers Evaporator Model BAK 600 at the Thin Films Laboratory from the São Carlos Institute of Physics (IFSC-USP). Part of electrodes metallic deposition was also performed at the Brazilian Nanotechnology National Laboratory (LNNano) facilities, from Brazilian Center for Research in Energy and Materials (CNPEM, from Portuguese *"Centro Nacional de Pesquisa e Energia em Materiais"*). First, a 150 nm layer of aluminum was deposited over the substrate surface, followed by a 700 nm insulating silicon dioxide layer (microdevice I). An alternative to producing a C^4D microdevice with easy-way insulation is to use coverslips on the opposite side of electrode deposition (microdevice II). The average thickness of coverslips ranges from 0.13 to 0.17 mm (indicated by the manufacturer).

To remove adhesive masks from the substrates, electrodes were immersed in isopropyl alcohol for one hour, followed by a washing step using distilled water and posterior drying with compressed air.

Microchannels were produced by cutting a double-sided adhesive sheet (ARcare™ 90106), and a cut polyester slide was employed for sealing the microdevice. Microchannels were designed as a straight line (550 mm × 0.5 mm) with circular inlets (1 mm i.d.), presenting 58 µm of average depth.

Copper wires were attached to the device's electrical contacts with conductive silver paste, followed by 24 h of drying at room temperature. Next, intravenous infusion microtubes (BD Asepto® 21 GA) and pipette tips were adapted to create flow inlets and outlets, using epoxy glue (Araldite™, TekBond, Brazil) at least 24 h of cure at room temperature. The fully assembled device is presented in Figure 15 (B).

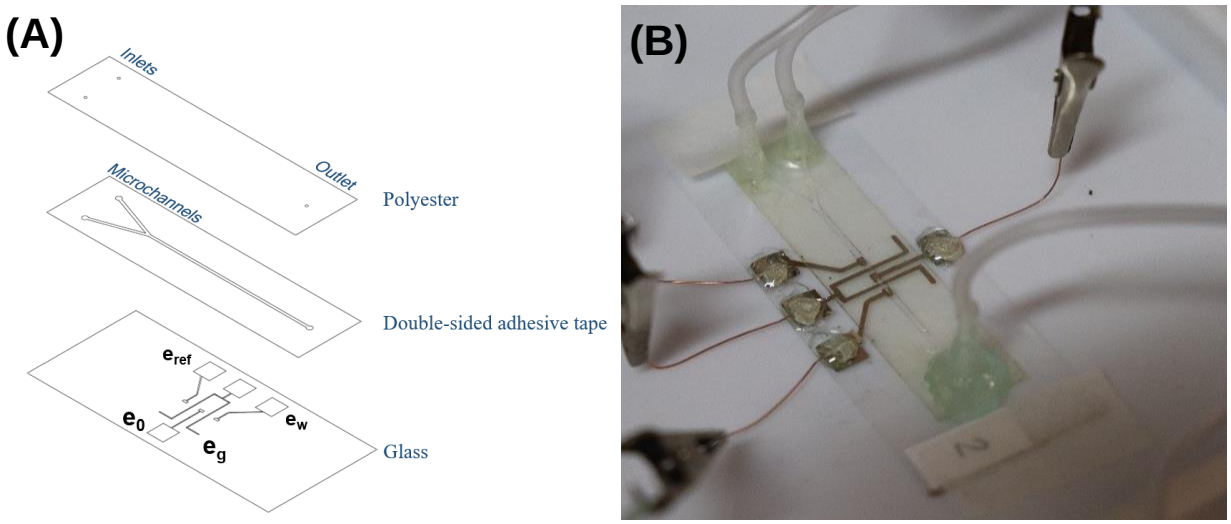
4.3.3 Setup for C^{4}D measurements

Electrochemical measures were performed using a setup constituted by: *(i)* syringe pump (New Era Pump Systems Inc.) with a 100 µL microsyringe (Hamilton™), *(ii)* homemade C^{4}D system (C^{4}D LAIA, Brazil) – construction is described in the paper from FRANCISCO & DO LAGO (2009); *(iii)* function generator (Tektronix CFG 250, Beaverton, USA), *(iv)* laptop computer to data acquisition. In addition, a homemade Faraday cage was used to minimize electromagnetic interferences in the C^{4}D system.

4.3.4 Optimization of C^{4}D signal for the proposed microdevice

A univariate study was carried out to optimize the C^{4}D signal towards amplitude and frequency parameters. First, the amplitude was varied from 0.25 to 2.00 V (frequency fixed at 500 kHz). Next, saline solution conductivity or ultrapure water conductivity had been measured by C^{4}D system with assistance of 50 µL min^{-1} flow to permeate each solution plug as follow: air gap; ultrapure water; air gap; 0.10 mmol L^{-1} NaCl; air gap; 0.25 mmol L^{-1} NaCl; air gap; 0.75 mmol L^{-1} NaCl; air gap; 1.00 mmol L^{-1} NaCl; air gap; and 2.5 mmol L^{-1} NaCl. Then, a similar assay was executed with frequency ranging from 300 to 1300 kHz for a sine function (amplitude fixed at 2.00 V). Finally, data acquired was evaluated by linear regression to evaluate the sensitivity of the device under each experimental condition.

Figure 15 - Microfluidic device diagram (A) and a picture of the device fully assembled (B).



Legend: $\mathbf{e_0}$ - excitation electrode; $\mathbf{e_{ref}}$ - reference electrode; $\mathbf{e_w}$ - working electrode; $\mathbf{e_g}$ - ground electrode.
Source: Letícia A. Marques (2021).

4.3.5 Analytical validation of the microdevice

As a proof of concept, microdevices were submitted to an analytical validation using standard saline solution to observe their analytical performance. A linear range from 0.10 to 2.50 mmol L^{-1}, including eight concentration levels, was constructed for three different microdevices. Each concentration level was measured in triplicate by permeating 10 µL of NaCl solution, and each solution plugged into air gaps (flow rate of 50 µL min^{-1}). In addition, blank solution (ultrapure water) was also measured in triplicate for each linear range.

Towards assessing the sensitivity values, the slopes of the analytical curve were normalized through division by electrodes area, apprising C^4D signal change due to analyte concentration variations.

LOD was evaluated through ratio calculation of 3 times the standard deviation of 10 blank measurements by the analytical curve slope. In its turn, LOQ was taken as the lowest calibration point of the analytical curve with appropriate repeatability (RSD < 5%) and accuracy (recovery rates varying from 80 to 120%).

To evaluate precision, repeatability was determined for 10 measurements at the medium concentration (1.50 mmol L^{-1} NaCl) for each microdevice. Additionally, reproducibility was assessed at medium concentration (1.50 mmol L^{-1} NaCl) measurements carried out for three distinct microdevices on different days.

4.3.6 Biological functionalization using anti-BSA approach and C^4D measurements

The system consisting of bovine serum albumin (BSA) and its respective antibody (BSA/anti-BSA model) was chosen to evaluate the biological functionalization of the surface of the electrode on the proposed microdevice.

For these experiments, microdevices with the coverslip as dielectric insulator (Microdevice II) were assembled on the day of biological functionalization execution. First, a daily fresh piranha solution 3:1 (H_2SO_4:H_2O_2) was prepared and used to clean the glass coverslip surface (opposite side of electrodes deposited). Previously prepared, the microchannel conjunct with inlets/outlets connections and polyester covering were then aligned to the cleaned electrodes. After microdevice assembling and before biofunctionalization, a C^4D measurement using PBS 1× buffer was performed.

At the beginning of the biological functionalization, the glass surface over the working electrode area was cleaned using piranha solution (3:1 H_2SO_4:H_2O_2) (step 1), followed by water washing. Then, 10 µL of 2% APTES solution (in ethanol) was incubated over the working electrode by manual alignment for 5 min (step 2), followed by gentle washing with 30 µL of

ethanol. Glutaraldehyde solution at 2.5% (v/v) was used to incubate the working electrode for 120 min (step 3), with a posterior 30 µL of ultrapure water solution washing. Then, it was incubated with 10 µL of a 200 µmol L^{-1} anti-BSA for 60 min (step 4), followed by PBS 1× (pH 7.3) buffer gentle washing. Finally, a 10 µL solution plug of 1% (w/v) BSA solution was incubated over working electrode for 30 min (step 5), followed by PBS solution washing. Between each incubation step, it was executed C^4D measurements of 10 µL plugs of PBS buffer (in triplicate).

4.3.7 Biological functionalization using aptamer-ligand approach.

The base sequence to obtain a specific aptamer for DDT molecule is described by 5'–TCCAGCACTCCACGCATAACGAATTGTGCTCAATGCGCCCCTGCAGTGAATGTGGAATTTGTTATGCGTGCGACGGTGAA–3', which was obtained from Zhang and collaborators study (2020). The 5'-end of aptamer was synthesized with an amino-terminal modification. The oligonucleotide sequence was obtained by Systematic Evolution of Ligands by Exponential Enrichment (SELEX) process from Extend Biotecnologia (Paulínia, São Paulo – Brazil) followed by HPLC purification. The aptamer contains 80 base pairs, presenting an average molecular weight of 24,761 kDa.

To obtain aptamer solutions, a tris-EDTA (TE) buffer was prepared with 10 mmol L^{-1} tris, 0.1 mmol L^{-1} EDTA, at pH 8.0, followed by autoclave sterilization at 120 °C for at least 20 min. First, the lyophilized aptamer was resuspended according to the manufacturer's instructions to provide a 100 µmol L^{-1} solution. Next, a dilution was performed to obtain a 10 µmol L^{-1} aptamer; 5 µL aliquots were made and kept at −20 ºC. Aptamer resuspension and dilution steps were performed in a laminar flow cabinet to avoid contamination of the biological matter. Then, on the analysis day, 45 µL of TE buffer was added into an aliquot at 10 µmol L^{-1} of aptamer, resulting in a 1 µmol L^{-1} working solution.

As described in section 4.3.6, microdevices were fully assembled on the day of the experiments. A series of incubation was performed to create a biological layer functionalized over the working electrode. The first step of this process was clean glass surface using piranha solution (2:1) (step 1). Then, ten microliters of each following solutions were infused in the microchannels over the working electrode: 2% ethanolic solution of APTES for 5 min (step 2); glutaraldehyde solution at 2.5% v/v for 120 min (step 3); aptamer solution at 1 µmol L^{-1} (step 4); DDT solution at 1 µg mL^{-1} for 30 min (step 5). Before the first incubation (clean electrode) and after each incubation step, it was carried out C^4D measurements of 10 µL plug of TE buffer (in triplicate).

4.3.8 Data analysis

Raw C^4D data were treated using smoothing of OriginLab™ (Northampton, MA, USA). Linearity data were assessed by ActionStat™ Pharma (Estatcamp, São Carlos – SP, Brazil).

4.4 Results and discussion

4.4.1 Fabrication characteristics, design aspects, and basic performance of the microdevice

Glass coverslips were chosen to construct microfluidic platforms for many reasons. First, glass usage as a microdevice substrate is common to found enough microfabrication options in the literature. From a chemical point-of-view, glass is an amorphous ceramic material, providing a rigid and robust surface to build a microdevice platform. In addition, glass is an inert material, offering great compatibility with biological samples, besides its optical transparency at the visible region of the spectrum, which is essential to CE applications (REN, ZHOU, WU, 2013; GOZOLAR *et al*., 2020). Glass is an easy-to-handle substrate amenable to the metal deposition with a fair and reasonable cost-benefit, and finally it provides a natural insulating, allowing that coverslip itself could be used as a dielectric layer of C^4D microdevices. These characteristics explain why glass-based microdevices have a prominent place between classic substrates for microfluidic platform fabrication.

The Y geometry was shown to be proper to whole solution flow through the microchannel, while air plug permeation promoted the cleaning of the channel with appropriate baseline signal return. A straight-line microchannel geometry was also tested in this work, showing an efficient solution flowing. Before each analysis, there was no leaking in the microdevices or bubbles formation into the microchannel.

Regarding detailing the microdevice dimensions, both excitation electrode, and working electrode presented a total area of 1.5 mm^2. The average depth of microchannels is 242.42 µm. ARcare™ 90106 is a pressure-sensitive adhesive made of polyester combined with MA-69 acrylic hybrid medical-grade, having a total thickness of 142.24 µm (being 25.4 µm of polyester film, and two sides of the medical-grade adhesive, with 58.42 µm each one) (KRATZ *et al*., 2019).

A cost production evaluation for these microdevices was executed. The total cost is US$1.23 per unit of the microchip (it does not include analysis cost). A detailed description of costs (reagents, materials, and hours of equipment used) is shown in our recent publication (TAKEKAWA *et al*., 2021). Considering the desirable cost of each POC device below US$5

(SHARMA *et al*., 2011), when the best situation is the cost of pennies, the presented microchips are appropriate.

4.4.2 Data of C^4D excitation signal optimization

Amplitude optimization data are shown in Table 12 and Figure 16 (A). For 0.25, 0.50, and 0.75 V amplitudes it was not observed considerable conductivity differences between the lower concentrations of NaCl solution and blank. Also, higher concentrations of NaCl showed to be not as proportional to the concentration as expected. So then, despite data collection for this excitation signal amplitude, we choose not to use it at the linear fitting of NaCl conductivity.

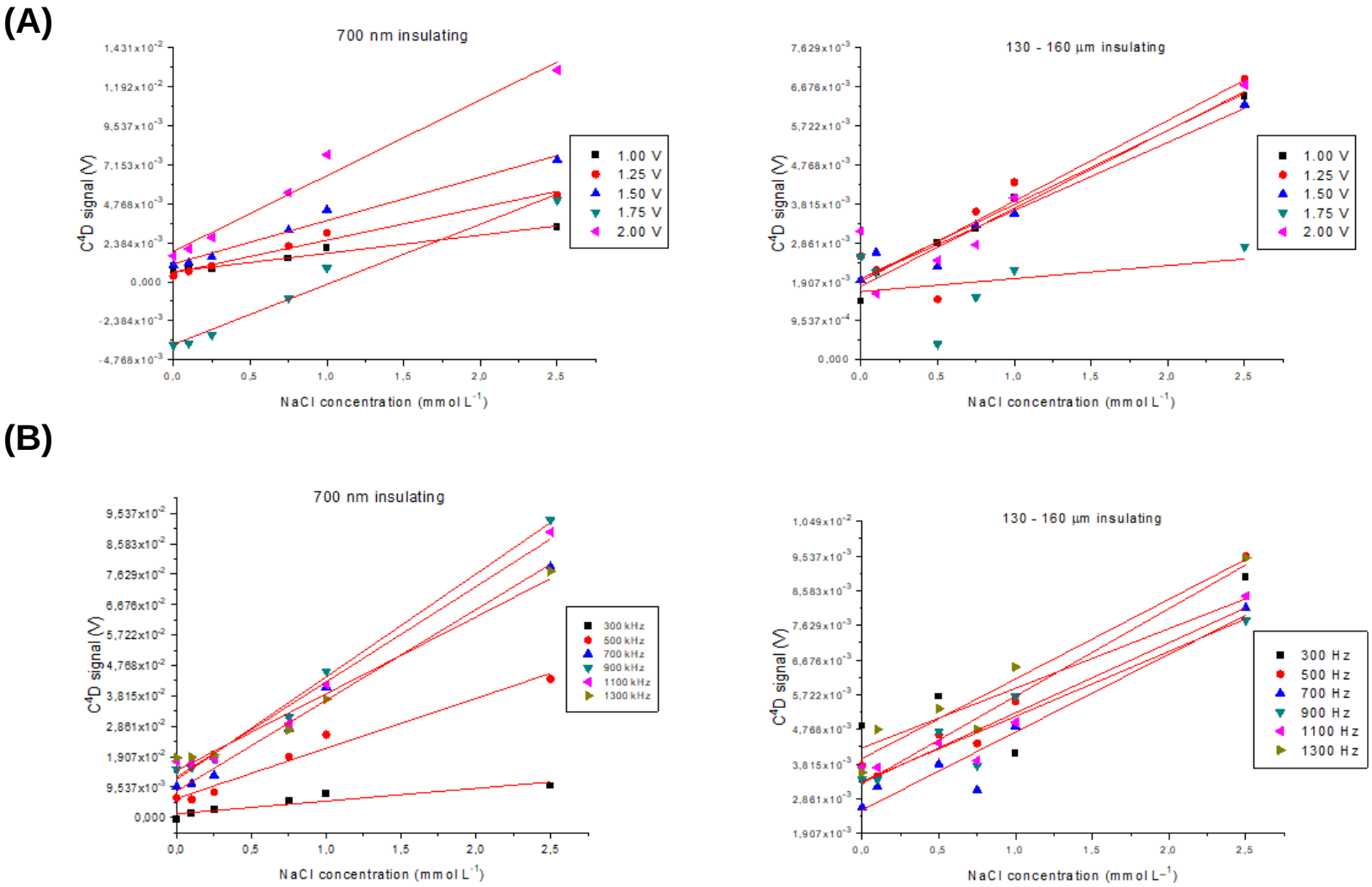
In this way, it is notable that conductivity linear fittings presented for the microdevice I (700 nm of insulating layer) presented a higher sensitivity difference with the amplitude increasing from 1.0 to 2.0 V. On the other hand, the microchip with thicker insulator did not show an expressive difference for amplitudes evaluated; moreover, the amplitude of 1.75 V did not fit well to the linear modeling, presenting a low determination coefficient (R^2). It would be possible to increase the sensitivity of C^4D using higher voltages; however, the employed C^4D systems operate ideally at 2.0 V of voltage. Higher values of voltage may saturate electronics of the system (TAKEKAWA *et al*., 2021). Therefore, the best voltage condition was 2.0 V for the microdevice I with a 700 nm insulator layer.

We can readily observe that C^4D measurements obtained for 1.0 V and 1.50 V presented the best linear fittings and higher slope values for the microdevices with a thicker insulator layer. Thus, the best voltage condition to C^4D operation for the microdevice II was set at 1.0 V for the following measures.

Table 12 - Linear fitting of the C^4D signal in the wave amplitude optimization assay

Parameter	**Wave amplitude (V)**				
	1.00	1.25	1.50	1.75	2.00
	Microdevice I (700 nm insulating)				
Intercept	6.25E-03	5.53E-03	1.09E-02	-3.83E-02	1,86E-02
Slope	1.12E-02	1.99E-02	2.65E-02	3.68E-02	4,63E-02
R^2	0.9707	0.9765	0.9760	0.9728	0.9719
	Microdevice II (130-160 µm insulating)				
Intercept	1.80E+02	1.91E+02	1.98E+02	1.66E+01	1.98E+02
Slope	1.90E+02	1.97E+02	1.66E+02	3.20E+00	1.81E+01
R^2	0.9793	0.8228	0.9460	-0.1074	0.8260

Figure 16 – Univariate optimization assays from the C^4D excitation signal amplitude (A) and frequency (B)



Legend: In part (A) of the figure, frequency fixed at 500 kHz; in part (B), amplitude held at 2.00 V; both optimizations were performed at 50 µL min^{-1} of flow rate.
Source: Letícia A. Marques (2021).

Table 13 and Figure 16 B show linear fittings data of the C^4D signals as a function of NaCl concentrations for distinct frequencies applied. Again, the thin insulator layer of the microdevice presented better linear fittings (R^2 were higher than 0.99, except for 300 kHz) than the thicker dielectric layer microchip. The best linear fitting of the coverslip microchip was obtained at 500 kHz, presenting an R^2 = 0.9585, which is below the second-worst R^2 value between those achieved for the thin layer microchip. For the microdevice I, a centered sensitivity is observed on 900 kHz frequency. However, this frequency condition was not as efficient as the 700 kHz condition on the discrimination of NaCl solution low concentrations. Also, it is remarkable that frequency variations were more expressive to the microdevice with 700 nm insulator than to the thicker one.

Table 13 - Linear fitting of the C^4D signal in the frequency optimization assay

Parameter	**Frequency (kHz)**					
	300	500	700	900	1100	1300
	Microdevice I (700 nm insulating)					
Intercept	1.24E-03	6.06E-03	8.34E-03	1.20E-02	1.27E-02	1.48E-02
Slope	3.96E-03	1.57E-02	2.84E-02	3.22E-02	2.99E-02	2.40E-02
R^2	0.8282	0.9650	0.9902	0.9895	0.9789	0.9726
	Microdevice II (130-160 µm insulating)					
Intercept	4.26E+02	3.28E+02	2.55E+01	3.34E+01	3.29E+02	3.97E+02
Slope	1.64E+02	2.41E+02	2.15E+02	1.79E+02	1.93E+02	2.19E+02
R^2	0.5288	0.9585	0.9138	0.8814	0.9157	0.9188

Therefore, optimum conditions for the C^4D excitation signal obtained in the univariate study were 700 kHz and 2.00 V for the microdevice I and 500 kHz and 1.00 V for the microdevice II.

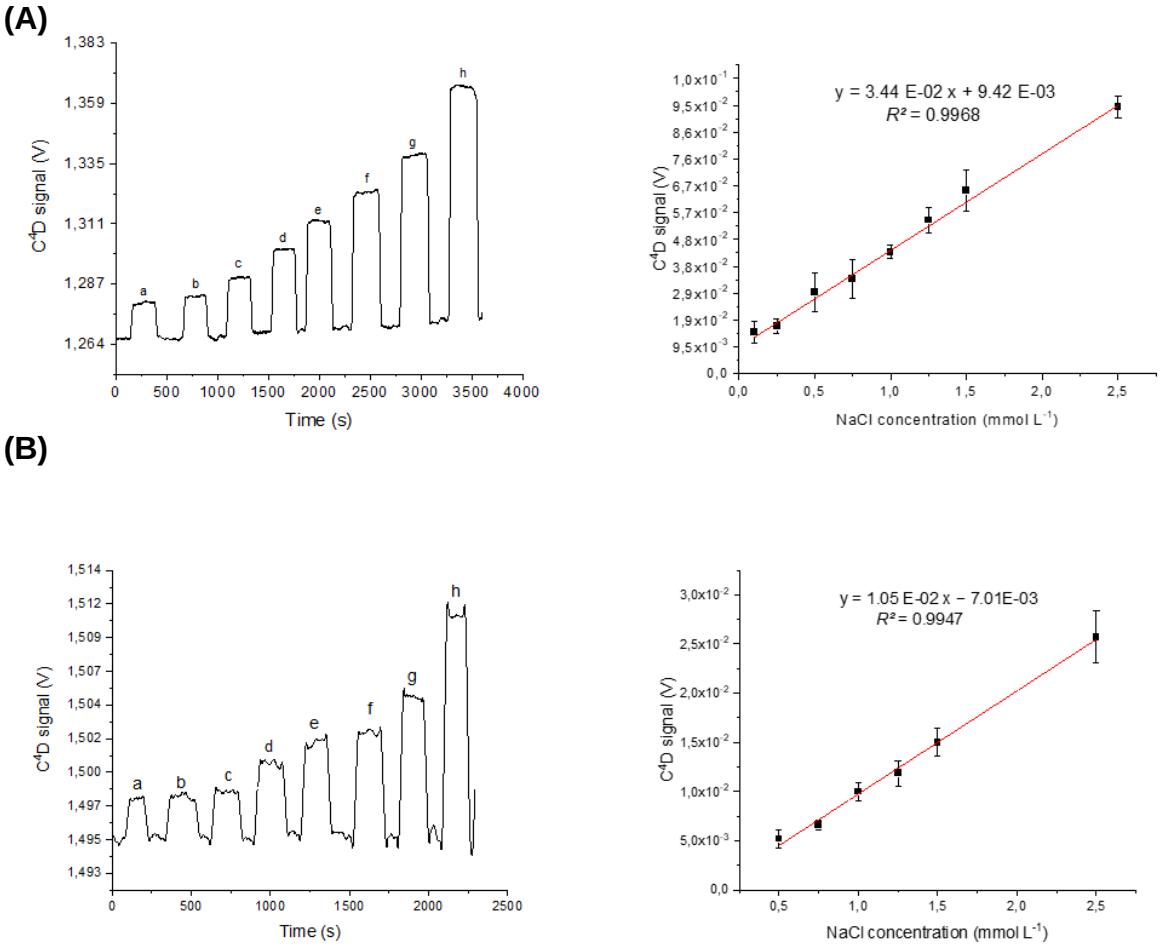
4.4.3 Analytical performance of the proposed microdevices

Figure 17A shows C^4D signals for NaCl solutions of increasing concentration as a function of acquisition time. It is noticed that increasing NaCl concentrations, enhance ionic species into solution; consequently, an expected conductivity signal increase. In its turn, linear fittings of the analytical curves are shown in Figure 17 B.

Linearity was achieved from 0.10 to 2.50 mmol L^{-1} for the thin insulator microdevice and from 0.50 – 2,50 mmol L^{-1} for the thicker one. This is stated since the determination coefficient (R^2) for both microchips was higher than 0.98. LOD and LOQ values are shown in

Table 14 for both microdevices. Values of LOD and LOQ for the thin dielectric layer microdevice were 0.06 and 0.10 mmol L^{-1}, respectively, which are lower than those obtained for the thicker insulator microdevice as expected. Comparing slopes and LOQ values, we noticed that microdevice I was more efficient in analytical parameters for C^4D signal than microdevice II.

Figure 17 - C^4D signals for NaCl solutions and linear fitting of the analytical curve using for microdevice I (A) and microdevice II (B).



Legend: Concentrations of NaCl solutions (**a**) 0.10; (**b**) 0.25; (**c**) 0.50; (**d**) 0.75; (**e**) 1.00; (**f**) 1.25; (**g**) 1.50; and (**h**) 2.50 mmol L^{-1}.
Source: Letícia A. Marques (2021).

The microdevice I, the thin dielectric layer one, showed excellent precision, with RSD values lower than 1.6% for both intraday precision and reproducibility assays. For the microdevice II, the thicker one, RSD values were 3.72% for intraday precision, while 5.78% of RSD was obtained for reproducibility. These results enable us to affirm, for both microchips,

that the microfabrication process presents good precision, and there is an excellent agreement between different conductivity measurements.

Nevertheless, precision data indicates greater precision in the microdevice I (700 nm of insulating layer) than microdevice II (coverslips); it can be assigned to the better reproducibility of thin SiO_2 layer deposition, performed in the laboratory, compared to the commercial production of glass coverslips. We must consider that used coverslips were originally designed and fabricated for microscopy usage, where thickness variations from 30 to 60 µm are much more acceptable for a batch of coverslips than would be for thin-film coverings.

Table 14 – Analytical validation results for the C^4D signal of the NaCl solution

Parameter	**Insulation layer**	
	700 nm *(Microdevice I)*	130 -160 µm *(Microdevice II)*
Operational wave parameters	2.0 V; 700 kHz	1.0 V; 500 kHz
Linear range (mmol L^{-1})	0.10 - 2.50	0.50 - 2.50
Intercept (V)	9.42E-03	-7.01E-03
Slope (V L $mmol^{-1}$)	3.44E-02	1.05E-02
Sensitivity (S $mmol^{-1}$)	2.29E-02	7.00E-03
R^2	0.9968	0.9947
LOD (mmol L^{-1})	0.06	0.26
LOQ (mmol L^{-1})	0.10	0.50
Intraday precision *(n=10)*	1.52%	5.28%
Intermediate precision *(n=3)*	1.58%	3.72%

Studies reported a significant gain in analytical signal when the thickness is decreased from micro to nanoscale. Indeed, we noticed that, for the proposed microdevices I and II, insulation thickness decreasing from 130-160 µm to 700 nm improves sensitivities around 3.3 times (from 7.00 to 22.90E−3 V). Still, regarding the dielectric layer, it is important to maintain effective insulation; otherwise, the high electric field applied in C^4D measurements could damage the detector's electronic circuit (COLTRO *et al*., 2014).

4.4.4 Immobilization aspects of biosensors and obtained C^4D data

For both biorecognition approaches, daily assembling of microdevices was performed in order to assure the best cleaning of glass coverslip surfaces as possible. Clean-up step using piranha solution is responsible for silanol exposure onto glass coverslips surface. Then, it begins a silanization reaction using APTES. The first step of silanization is hydrolysis, where it will be formed reactive silanol groups. Condensation is the second part of the silanization reaction;

it was carried out by the APTES solution incubation, resulting in siloxane bonding over the glass surface. This APTES coating over glass surfaces occurs due to the binding between amino groups from the organosilane and the glass surface silanes. Following the third step, hydrogen bonds were formed with −OH groups from the glass. Finally, it is formed a covalent bond Si-O-Si between silicon of APTES and the silicon on the glass surface, with loss of water molecules (GUNDA *et al*., 2014).

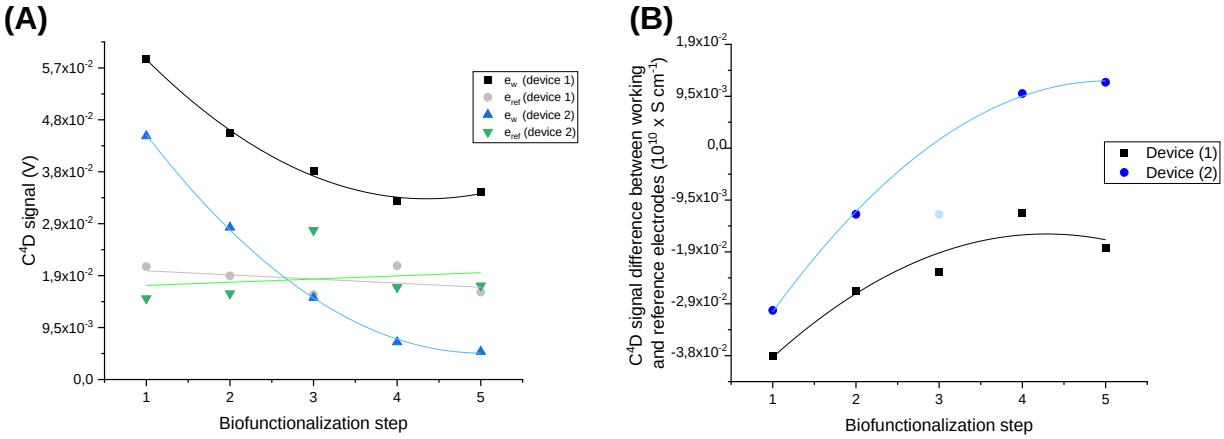
In the case of antibody-antigen based microdevice, it was first employed EDC/NHS to the conjugation protocol of anti-BSA. This strategy was adopted since EDC/NHS protocols are well-established for antibodies and proteins, comprehending applications from biosensors to nanoparticles covering. From a chemical perspective, EDC is considered a crosslinked capable of couple carboxyl groups to primary amines. It is initially obtained an active ester intermediate, allowing a reaction with strong nucleophiles, such as a primary amine. Later, NHS undergoes a nucleophilic substitution, forming a reactive NHS ester. Considering the contact between primary amine groups from an antibody (or another protein) and ester, a covalent binding will be established (JAZAYERI *et al*., 2016; WICKRAMATHILAKA & TAO, 2019). Thus, it was possible to immobilize anti-BSA over the working electrode surface and then perform a specific detection of BSA.

Once microdevice presented an expected behavior using EDC/NHS protocol, another crosslinker compound, glutaraldehyde, was tested to observe it would be appropriate to antibody immobilization, aiming to crosslinker protocol transference to the aptamer. Glutaraldehyde is considered a homobifunctional crosslinker once it presents aldehyde groups at both molecule's extremities. Because of this feature, glutaraldehyde is widely applied as a crosslinker of biomolecules immobilization. One aldehyde reacts with the amine of the silane layer. In contrast, the other aldehyde group reacts to the biomolecule extremities, such as amine groups of antibodies or *N*-extremity from modified aptamers. Thus, glutaraldehyde-biomolecule linking is established by forming covalent bonds involving amine-glutaraldehyde-amine (GUNDA *et al*., 2014).

Figure 18 presents anti-BSA based biosensor data. An exponential decreasing profile of working electrode C^4D signal is noticed in Figure 18 (A) after biofunctionalization steps (described in section 4.3.6). The signal difference between working and reference electrodes is shown in Figure 18 (B). We can observe an increasing trend in the values of conductivity difference, also showing an exponential shape for both devices evaluated. It is also important to highlight that device 1 cross over zero-line for the conductivity difference. The trend noticed is to increase C^4D signal difference after performing biofuncionalization steps 4 and 5. In

general, signal suppression performance is expected in biosensors, including aptasensors, once biomolecular recognition diminishes current flow due to the increase in double-layer capacitance (MING *et al*., 2020).

Figure 18 – C^4D anti-BSA based biosensors conductivities measurements after each incubation step (A) and the difference between working and reference electrode signals (B).



Legend: Biofunctionalization steps were coded as cleaning electrode (1), 2% APTES for 5 min (2); 2.5% (v/v) glutaraldehyde for 120 min (3); 200 µmol L^{-1} anti-BSA for 60 min (4); and 1% (w/v) BSA for 30 min (5). In part B of the figure, light blue point (respective to device 2 data) was not considered to polynomial fitting. $\mathbf{e_{ref}}$ - reference electrode; $\mathbf{e_w}$ - working electrode.
Source: Letícia A. Marques (2021).

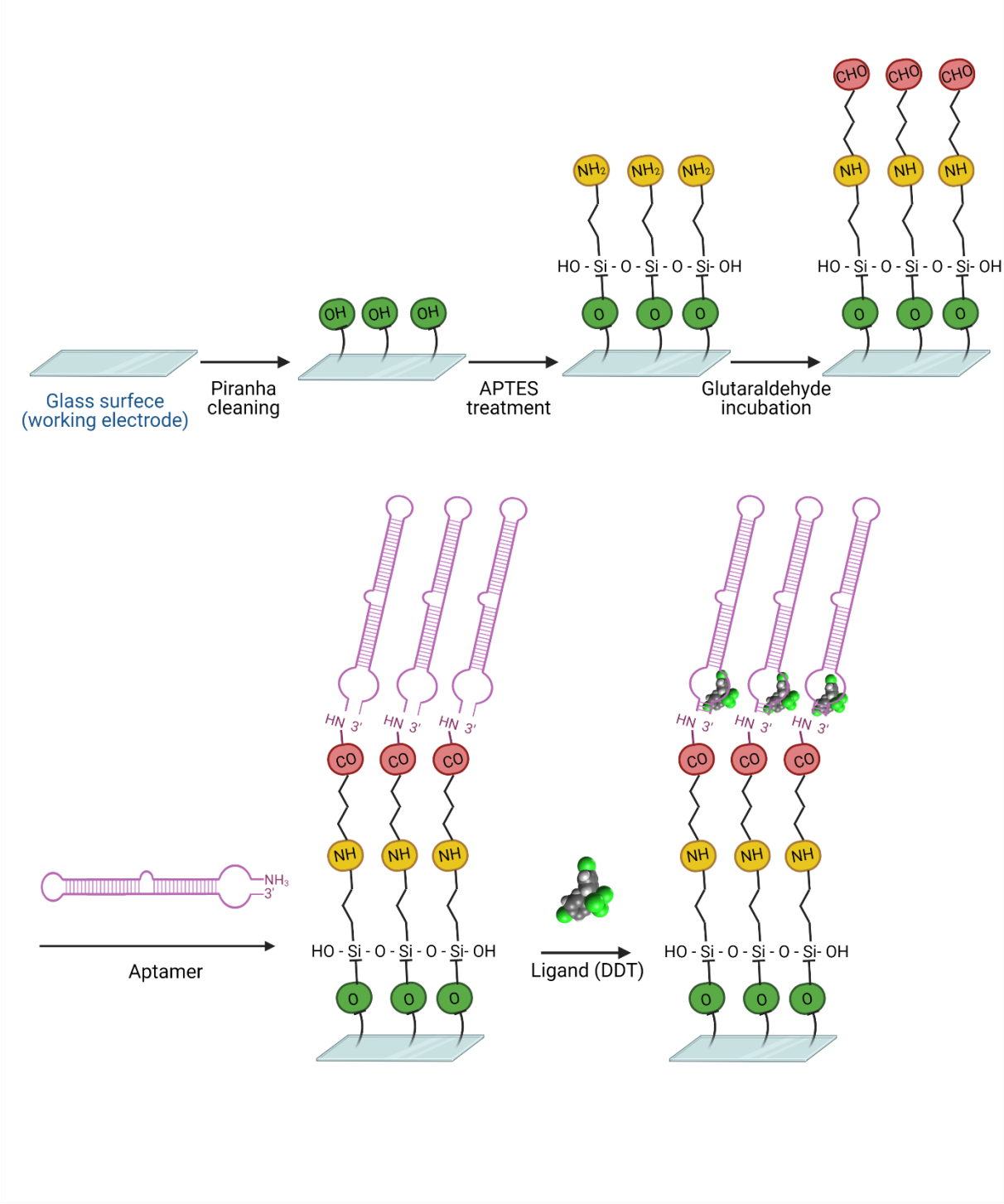
After biosensor performance verification using BSA/anti-BSA model, microdevices were produced to receive aptamer-ligand as biorecognition system. A complete scheme of the aptasensor surface functionalization is presented in Figure 19.

Aptasensor data for TE buffer measurements are represented in Figure 20. There are similar behaviors observed for the aptasensor relative to the anti-BSA biosensor. First, the C^4D signal produced by the working electrode has a diminished trend for both devices appraised, while reference electrodes reveal constant signals. Also, a crescent exponential tendency is observed for the conductivity difference between e_{ref} and e_w. However, the growing tendency has a convex polynomial profile in aptasensor, which is different from the growing concave tendency observed for the anti-BSA biosensor (Figure 18).

The interaction between the DDT ligand and its respective aptamer promotes a remarkable C^4D signal reduction. Therefore, it is possible to affirm that the proposed aptasensor can be used as a semi-quantitative device for a fast and easy evaluation of DDT presence in TE solution. It is interesting to highlight that a sequence of three measures can be achieved in approximately 5 minutes using our microfluidic platforms. Furthermore, our aptasensors

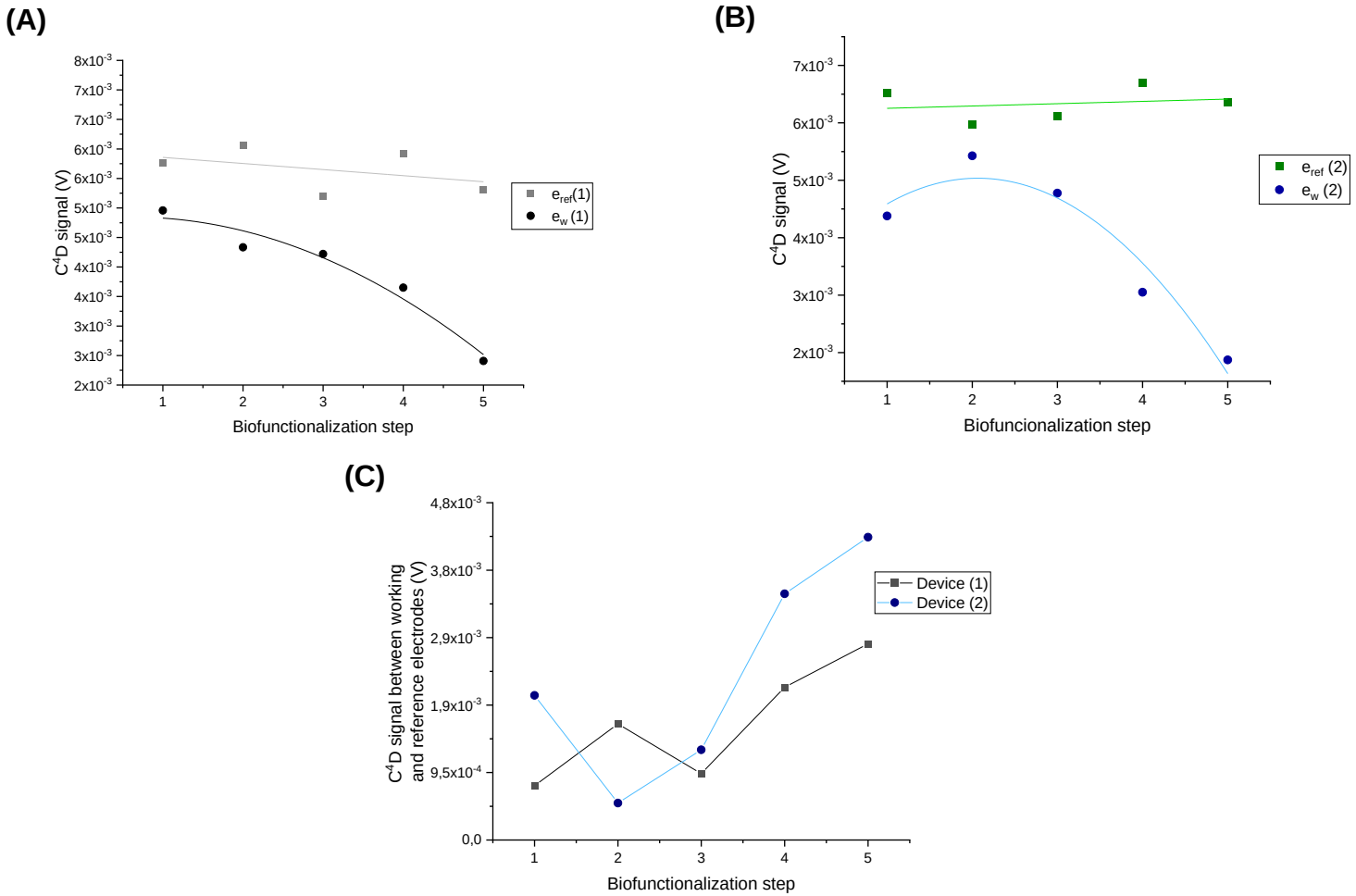
showed to be appropriate even for quantitative purposes, which may enable this aptasensor evaluation as a quantitative microdevice for DDT determination in food or environmental samples.

Figure 19 – General scheme of the aptasensor surface functionalization



Source: Letícia A. Marques (2021), created with BioRender.com.

Figure 20 – C^4D aptamer-based biosensor conductivities measurements after each incubation step (A and B) and the difference between working and reference electrode signals (C).



Legend: Biofunctionalization steps were coded as cleaning electrode (1), 2% APTES for 5 min (2); 2.5% (v/v) glutaraldehyde for 120 min (3); 1 µmol L^{-1} aptamer for 60 min (4); and 1 µg mL^{-1} DDT for 30 min (5). e_0 - excitation electrode; e_{ref} - reference electrode; e_w - working electrode.
Source: Letícia A. Marques (2021).

4.5. Conclusions

A novel low-cost protocol to fabricate glass-based microchips was established, providing flexible microchips for analytical appliances.

Two different insulation layers' microdevices were evaluated – as presumed, the thinner one presented a greater sensitivity over the thicker one. Notwithstanding, microchip using coverslips thickness as the dielectric layer was competent to achieve conductivity measurements from ionic solutions to biomolecules. A substantial consequence of this is the possibility to perform good quality C^4D analysis using not so thin dielectric layers, offering a fast and low-cost microfabrication protocol.

The analytical performance of the microdevice for the NaCl solution was successfully demonstrated in the validation. Also, this microchip adaptability enables its application as a biosensor, whether using antibody-antigen or aptamer-ligand systems to biorecognition.

Finally, it was possible to produce for the first time a C^4D-based aptasensor for the semi-quantitative determination of DDT, representing significant progress in the small molecule detection in the biosensors field.

GENERAL CONCLUSIONS

This thesis demonstrated that monitoring of PCBs and OCPs in foodstuff is a crucial research line to be maintained. It is essential to highlight the necessity of developing this research line by professionals from different areas - as analytical, electrochemical chemists, food and environmental engineers, biologists, veterinarians, medics, statisticians, etc.

Analytical methods proposed by this work are a step for the consolidation of this research line by IQSC. Analysis of organochlorine compounds using miniaturized QuEChERS followed of GC-MS/MS analysis takes at least 1 (one) hour per sample (around 40 min to sample prep + 20 min of instrumental analysis), while microdevices can perform three C^4D measurements in approximately 5 minutes, indicating an expressive enhancing in analytical frequency.

The development of an official methodology for the extraction of PCBs and OCPs from raw milk was also initiated in partnership with LFDA-MAPA. Although presential activities have been paused, there is an intention to maintain this partnership with MAPA, because of its mutual benefits generated.

Regarding to microdevices, the present data demonstrate the feasibility of its use as biosensors to small molecules detection as PCBs and OCPs. A paper about the low-cost fabrication of this C^4D-based microdevice is already published in Electrophoresis, using several results presented in this thesis. Of course, there are expectations to perform a series of experiments with DDT aptasensor, particularly validation – contemplating analytical curve, intraday and intermediate precision, and interference assays, essential to producing a higher impact publication of these results.

PERSPECTIVES

Considering the advances reached by this work, there are interesting perspectives for future research:

i. The miniaturized QuEChERS method for PCBs extraction from milk samples can be applied for the analysis of polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and flame retardants compounds, as polybrominated diphenyl ethers (PBDEs), and other brominated or organophosphate flame retardants.

ii. Perform quantitation of PCBs and OCPs through isotopic dilution analysis (IDA).

iii. Improve C^4D-based microfluidic device sensitivity at least 100 times to reach LOQ obtained by the GC-MS/MS method, allowing microchip usage as a screening device.

iv. Use other biorecognition systems in the proposed microchip to create new types of biosensors, e.g., artificial antibodies, DNA origami, etc.

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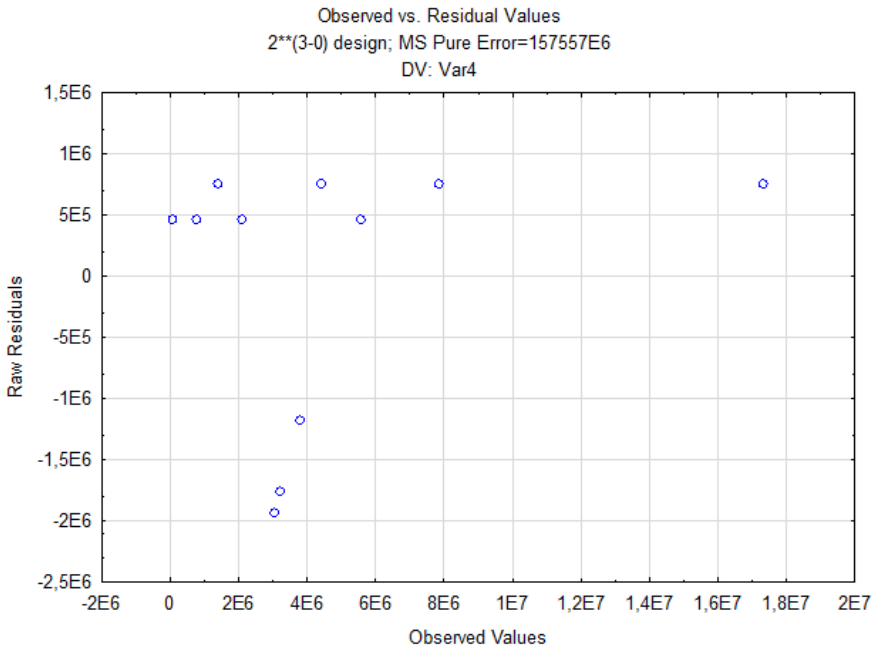
APPENDIX A

Supplementary Information

Table A 1 – Detailed nomenclature of main OCPs

Abbreviation	IUPAC nomenclature	CAS number
Dichlorodiphenylethanes		
DDT or p,p'-DDT	1,1'-(2,2,2-Trichloroethane-1,1-diyl)bis(4-chlorobenzene) or 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane	50-29-3
o,p'-DDT	1-chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]benzene or 1,1,1-trichloro-2-(o-chlorophenyl)-2-(p chlorophenyl)-ethane	789-02-6
p,p'-DDE	1,1-Dichloro-2,2-bis(4-chlorophenyl)ethene or 1,1-dichloro-2,2-bis(p-chlorophenyl)-ethylene	72-55-9
o,p'-DDE or 2,4'-DDE	1,1-dichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)ethylene	3424-82-6
p,p'-DDD	1,1-Dichloro-2,2-bis(4-chlorophenyl)ethane	72-54-8
o,p'-DDD	1,1-dichloro-2-(o-chlorophenyl)-2-(p chlorophenyl)ethane	53-19-0
2,4-D	(2,4-dichlorophenoxy)acetic acid	94-75-7
2,4,5-T	(2,4,5-Trichlorophenoxy)acetic acid	93-76-5
MTX (methoxychlor)	1,1,1-Trichloro-2,2-bis(4-methoxyphenyl)ethane	72-43-5
Hexachlorocyclohexanes		
HCB - hexachlorobenzene	1,2,3,4,5,6-hexachlorobenzene	118-74-1
α-HCH (alpha-hexachlorocyclohexane) or α-lindane	α-1,2,3,4,5,6-hexachlorocyclohexane	319-84-6
β-HCH (beta-hexachlorocyclohexane) or β-benzenehexachloride (β-BCH)	β-1,2,3,4,5,6-hexachlorocyclohexane	319-85-7
LIN (lindane) or gama-hexachlorocycohexane (γ-HCH)	γ-1,2,3,4,5,6-hexachlorocyclohexane	58-89-9
HPX (heptachlor epoxide)	1,6,8,9,10,11,11-heptachloro-4-oxatetracyclo[6.2.1.$0^{2,7}$.$0^{3,5}$]undec-9-ene	1024-57-3
cis-chlordane (*cis*-CLD) or *alpha*-chlordane (α-CLD)	(1R,2S,3R,4S,6S,7S)-1,3,4,7,8,9,10,10-octachlorotricyclo[5.2.1.$0^{2,6}$]dec-8-ene	5103-71-9
trans-chlordane (*trans*-CLD)	(1S,2S,3R,4R,6S,7R)-1,3,4,7,8,9,10,10-octachlorotricyclo[5.2.1.$0^{2,6}$]dec-8-ene	5103-74-9
Cyclodienes		
Aldrin (ALD)	1,2,3,4,10,10-Hexachloro-1,4,4a,5,8,8a-hexahydro-1,4:5,8-dimethanonaphthalene	309-00-2
Dieldrin (DLD)	(1aR,2R,2aS,3S,6R,6aR,7S,7aS)-3,4,5,6,9,9-hexachloro-1a,2,2a,3,6,6a,7,7a-octahydro-2,7:3,6-dimethanonaphtho[2,3-b]oxirene	60-57-1
Endrin (END)	(1R,2S,3R,6S,7R,8S,9S,11R)-3,4,5,6,13,13-Hexachloro-10-oxapentacyclo[6.3.1.13,6.02,7.09,11]tridec-4-ene	72-20-8
Heptachlor (HEP)	1,4,5,6,7,8,8-Heptachloro-3a,4,7,7a-tetrahydro-4,7-methano-1H-indene	76-44-8
Bornanes and derivates		
Toxaphene	1,4,5,6,7,7-hexachloro-2,2-bis(chloromethyl)-3-methylidenebicyclo[2.2.1]heptane	8001-35-2
Dodecachlor or mirex	1,1a,2,2,3,3a,4,5,5,5a,5b,6-dodecachlorooctahydro-1H-1,3,4-(methanetriyl)cyclobuta[cd]pentalene	2385-85-5
Chlordecone	1,2,3,4,6,7,8,9,10,10-decachloropentacyclo[5.3.0.02,6.03,9.04,8]decan-5-one	143-50-0

Figure A 1 – Residues of 2^3 factorial design matrix of PCBs total peak area extracted from UHT whole milk using PDMS/DVB SPME fiber.



Source: Letícia A. Marques.

Section A1 – Detailed explanation about penalty points calculation for the Eco-Scale obtainment

In this section, there is a brief explanation of the instructions presented in the paper entitled "Analytical Eco-Scale for assessing the greenness of analytical procedures" from Gałuska and collaborators (2012). In this work, the authors proposed a quantitative scale, called Eco-Scale, for evaluating the greenness of a methodology. As previous cited in the Section 2.4.2, this scale ranges from 0 (inappropriate green method) to 100 points (excellent green method). To obtain the score of a procedure, penalty points are assigned to parameters of an analytical methodology when it is not in agreement with the ideal green analysis. Then, the Eco-scale score can be achieved as described by Equation I:

$$Eco - score = 100 - \left(\sum PP_{reagents} + \sum PP_{instruments}\right) \qquad \textit{Equation I}$$

Where $PP_{reagents}$ is penalty points for all reagents used, and $PP_{instrument}$ is the penalty points for all equipment utilized also considering the waste disposal.

To obtain instrumental penalty points, we must observe equipment's energy-consuming. It is obtained by the multiplication of equipment potency and time of usage. Then, it must be assigned penalty points to instruments as follow:

- ≤ 0.1 kWh per sample – 0 penalty point.
- ≤ 1.5 kWh per sample – 1 penalty point;
- > 1.5 kWh per sample – 2 penalty points.

The calculation of penalty points for reagents is based on the sum of the pictograms earned by the product amount and hazard. We can express as:

$$\sum PP_{reagents} = \left(Amount_{R_1} \times Hazard_{R_1}\right) + \cdots + \left(Amount_{R_n} \times Hazard_{R_n}\right) \qquad \textit{Equation II}$$

Where $PP_{reagents}$ is penalty points for all reagents used, R_1 is reagent 1, and R_n is the n-th reagent.

Regarding the amount of reagents, penalty points are assigned as follow:

- less than 10 mL (or g) - 1 penalty point
- from 10 to 100 mL (or g) - 2 penalty points
- more than 100 mL (or g) - 3 penalty points

Hazard is a sum of the product between the number of pictograms and signals words. So, total penalty points for a reagent can be described as follow:

$$\sum PP_{reagents} = \left(Amount_{R_1} \times number\ of\ pictograms_{R_1} \times signal\ word_{R_1}\right) + \cdots + \left(Amount_{R_n} \times number\ of\ pictograms_{R_n} \times signal\ word_{R_n}\right) \qquad \textit{Equation III}$$

Where $PP_{reagents}$ is penalty points for all reagents used, R_1 is reagent 1, and R_n is the n-th reagent.

Safety datasheet for a reagent includes a pictogram, which can present or not a signal word. It must be assigned 2 penalty points if "danger" was the signal word, and 1 penalty point if "warning" was the signal word. If there was no signal word, zero penalty points must be assigned.

Finally, Eco-scale's score will be achieved after considering each reagent (amount and hazard) and each step of an analytical procedure. We recommend the full reading of the paper from Gałuska et al. (2012) for more information.

Table A2 – Data obtained for 2^4 factorial design for the miniaturized QuEChERS to extract PCBs from UHT whole-milk

Run	**Variable**				**Response function**
	Sample volume	m MSQ1	m PSA	MeCN volume	Total peak area
1	–1	–1	–1	–1	137457
2	1	–1	–1	–1	327617
3	–1	1	–1	–1	184652
4	1	1	–1	–1	375873
5	–1	–1	1	–1	119098
6	1	–1	1	–1	177196
7	–1	1	1	–1	78173
8	1	1	1	–1	248808
9	–1	–1	–1	1	229416
10	1	–1	–1	1	808919
11	–1	1	–1	1	512589
12	1	1	–1	1	683302
13	–1	–1	1	1	296432
14	1	–1	1	1	731538
15	–1	1	1	1	285787
16	1	1	1	1	820995
17	0	0	0	0	493636
18	0	0	0	0	519331
19	0	0	0	0	424013

Equation A1 – Mathematic model of the 2^4 factorial design for the miniaturized QuEChERS

$$y = 392359{,}5 + 145665.2\,X_1 + 22656.7\,X_2 - 31362.5\,X_3 + 170006.6\,X_4 + 69400.9\,X_1X_4 \qquad R^2 = 0.9041$$

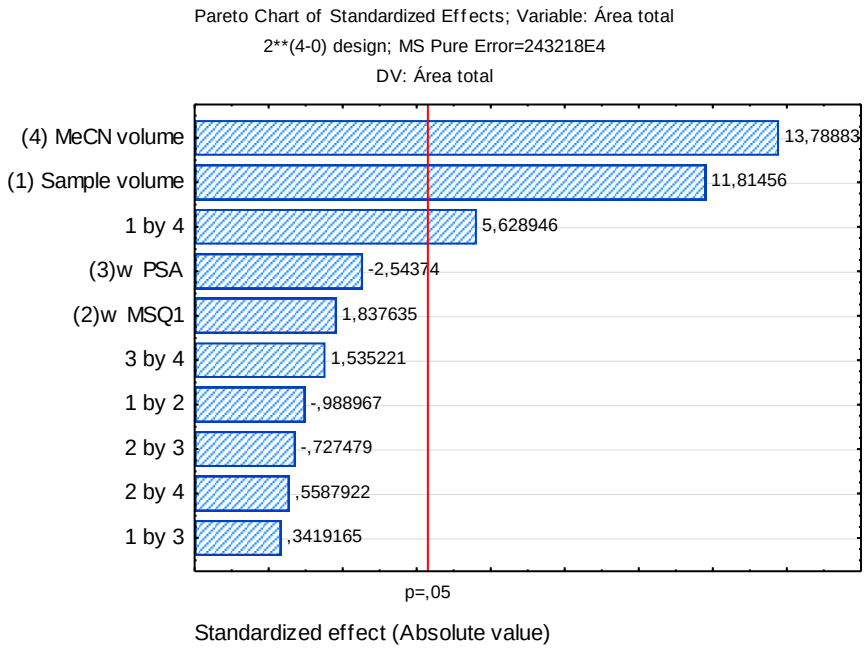
Where y is total peak area, X_1 is the sample volume, X_2 is the weight of MSQ1, X_3 is the weight of PSA, and X_4 is the solvent volume (MeCN).

Table A3 - ANOVA of the 2^4 factorial design for the miniaturized QuEChERS to extract PCBs from UHT whole-milk

Variable	*Square Sum*	*D.f*	*Square Average*	*F-test*	*p*
Curvature	2.674E+10	1	2.674E+10	10.993	0.080
(1) Sample volume	3.395E+11	1	3.395E+11	139.584	0.007
(2) w MSQ1	8.213E+09	1	8.213E+09	3.377	0.208
(3) w PSA	1.574E+10	1	1.574E+10	6.71	0.126
(4) MeCN volume	4.624E+11	1	4.624E+11	190.132	0.005
1 by 2	2.379E+09	1	2.379E+09	0.978	0.427
1 by 3	2.843E+08	1	2.843E+08	0.117	0.765
1 by 4	7.706E+10	1	7.706E+10	31.685	0.030
2 by 3	1.287E+09	1	1.287E+09	0.529	0.543
2 by 4	7.594E+08	1	7.594E+08	0.312	0.633
3 by 4	5.732E+09	1	5.732E+09	2.357	0.265
Lack of fit	5.377E+10	5	1.075E+10	4.421	0.195
Pure error	4.864E+09	2	2.432E+09		
Total	9.988E+11	18			

Legend: Df = degrees of freedom

Figure A 2– Pareto's chart for 2^4 model obtained for the miniaturized QuEChERS to extract PCBs from UHT whole-milk



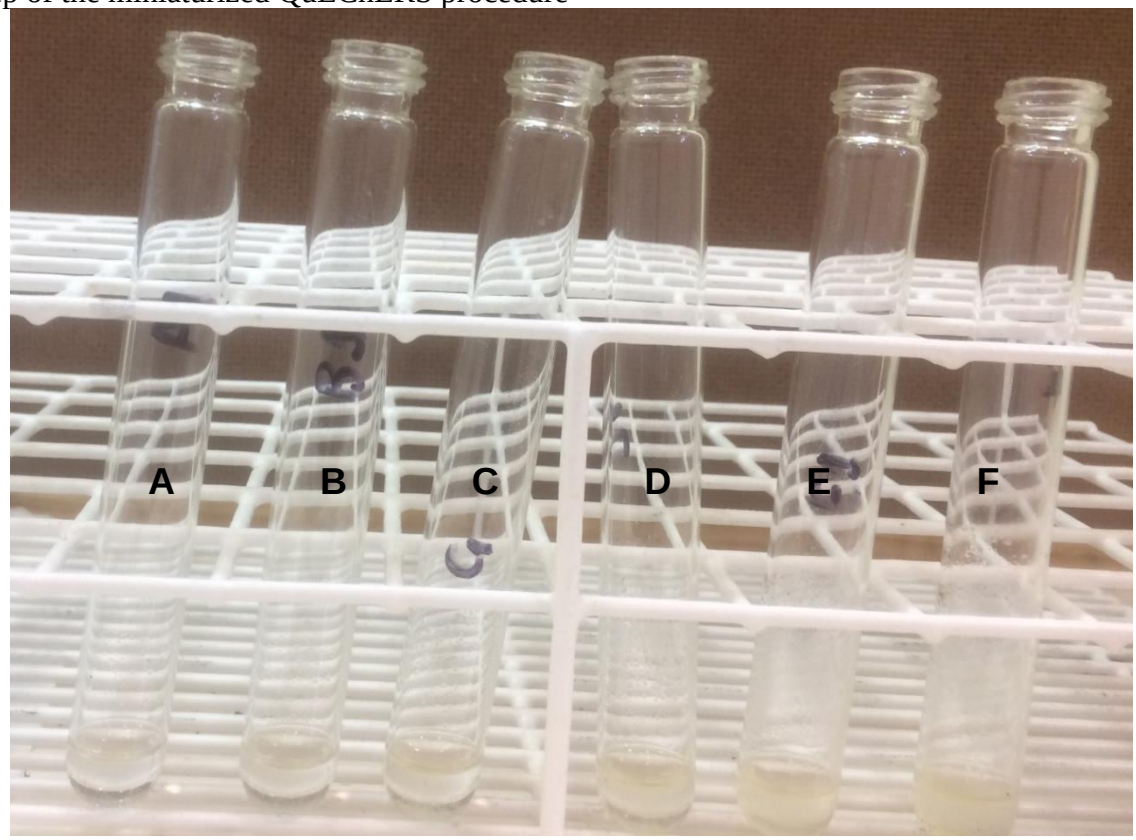
Source: Letícia A. Marques.

Table A4 – Results for walking over the surface from 2^4 factorial design model obtained for the miniaturized QuEChERS

Essay identification	**Essay**	**Experimental condition** (volumes)		**Total peak area**	**RSD%**
		Sample (µL)	MeCN (mL)		
A	Central point (CP)	1000	3	211160.2	13.4%
B	CP+1Δ	1425	4	321729.8	7.3%
C	CP+2Δ	1850	5	507476.0	9.5%
D	CP+3Δ	2300	6	519736.6	1.6%
E	CP+4Δ	2750	7	862771.3	30.9%
F	CP+5Δ	3125	8	866867.2	21.9%

Legend: In this test, weight of MSQ1 and PSA were held in 1.30 g and 100 mg, respectively.

Figure A3 – Supernatants of the samples from walking over the surface essay after clean-up step of the miniaturized QuEChERS procedure



Legend: A1 - central point (CP) essay; B1 - CP + 1 Δ; C1 - CP+2Δ; D1 - CP+3Δ; E1 - CP+4Δ; and F1 - PC + 5Δ.

Table A5 – Matrix effect for the extraction of PCBs from UHT whole-milk using a miniaturized QuEChERS procedure followed by GC- MS/MS analysis

Comparison test	***Square sum***			***p*-value**		
	Equality of intercepts	*Parellelism*	*Coincidence*	*Equality of intercepts*	*Parallelism*	*Coincidence*
PCB 28	8.70E+09	2.96E+12	5.99E+12	0.5300	7.5E-18	1.4E-24
PCB 52	3.63E+09	5.75E+11	1.12E+12	0.3476	2.7E-18	1.1E-24
PCB 101	1.08E+09	5.24E+11	1.07E+12	0.5497	1.6E-20	7.0E-28
PCB 118	6.73E+08	5.53E+11	1.15E+12	0.5456	9.1E-27	3.9E-35
PCB 153	1.10E+09	2.07E+11	4.07E+11	0.3351	1.2E-20	1.5E-27
PCB 153	1.56E+09	2.02E+11	3.89E+11	0.2701	1.4E-19	5.1E-26
PCB 156	2.76E+08	1.62E+11	3.33E+11	0.5158	1.8E-24	1.9E-32
PCB 170	9.28E+07	5.90E+10	1.22E+11	0.5374	3.4E-24	3.4E-32
PCB 180	2.21E+08	8.47E+10	1.72E+11	0.4370	1.0E-23	2.0E-31

APPENDIX B

Complementary project "*Pesquisa sobre Hábitos Alimentares para Estimar a Ingestão Diária de Leite*"

Relevance of assessing cow's milk consumption by children: In the human diet, consumption bovine milk usually begins as a replacement for breastfeeding. WHO recommends feed infants exclusively with breast milk at the first six months of life; for children under one-year of age, there is also a recommendation from the American Academy of Pediatrics to do not feed infants under one-year-old with raw, unmodified, or unpasteurized milk. Once breastfeeding is not possible or neither supply the nutritional requirements for the infants, it is popular to introduce bovine milk as a substitute for breast milk in children's diet. Indeed, cow's milk represents 83% of the global production of milk, which turns it the most common type of milk (BORTOLINI *et al*., 2013; MARTIN, LING, BLACKBURN, 2016; VERDUCI *et al*., 2019).

Objective: Conduct a questionnaire research on elementary schools to evaluate milk consumption in the infant population (6 to 10 years old) from São Carlos city (São Paulo state, Brazil).

Ethics approval: This part of the work was evaluated by Platform Brazil and Municipal Secretary of Education of São Carlos city, being further approved by the Research Ethics Committee (CAAE 65685317.9.0000.5504; n. 2.260.550) of Federal University of São Carlos (UFSCar). These documents are presented in Attachment A. This complementary study was carried out concomitantly to milk sample collection; if these milk samples presented PCBs contamination, it would be possible to evaluate children's non-occupational exposure to PCBs through milk intake.

Procedure: Four elementary schools of São Carlos city were selected for the application of the questionnaires. One class of each year from the 1st to 5th year of elementary school was chosen to be interviewed in each school, adding up to 240 interviewed children. Questionnaires were made using questions about age, gender, consumption or restrictions to milk or dairy products, quantities of milk cups ingested per week. The questionnaires were applied in two stages. In the first one, children were invited to join the research by explaining to them the research aim, benefits, and the way each child (and its parents or legally responsible) would enroll. Following, each child received one copy of the questionnaire, one copy of informed assent (TA – *Termo*

de Assentimento), and two copies of an informed consenting form (TCLE – *Termo de Consentimento Livre e Esclarecido*). They were instructed to take these documents home and answering them in the presence of the parents. After a 2- or 3-day interval, the second stage was executed by returning to the schools and recalling the documents. Those children who brought both TCLE and questionnaire assigned were taken into account to carry out weight and height measures.

Outcomes of children questionnaires about milk intake: A series of other interesting observations about Brazilian children milk consumption were obtained and they are presented in Table B 1. More information about physical characteristics as body weight, height, and body mass index (BMI) of the children interviewed are displayed by age and gender in Table B 2.

Table B 1– Average daily intake of whole-milk for the infant population

Age group	**N**	**% $N_{restriction}$**	**Average whole-milk consumption** (mL milk day^{-1})		
			UHT	*Pasteurized*	*Raw*
6 y.o.	43	11.6%	123.6	70.4	17.2
7 y.o.	47	4.3%	124.6	64.4	15.8
8 y.o.	54	1.9%	142.3	56.0	13.7
9 y.o.	49	12.2%	118.4	61.8	15.1
10 y.o.	47	6.4%	119.2	64.4	15.8
Total	240	7.1%	126.0	12.6	3.0

Legend: y.o. – years old; *N* – number of individuals; $N_{restriction}$ - the percentage of individuals with restriction to milk consumption (lactose intolerance, casein allergy, others).

Table B 2– Average physical characteristics (body weight, height, and body mass index) of the

Age group	**Average body weight** (kg)			**Average height** (m)			**BMI*** ($kg\ m^{-2}$)		
	Total	*Female*	*Male*	*Total*	*Female*	*Male*	*Female*	*Male*	
6 y.o.	21.76	22.68	20.31	1.24	1.24	1.23	14.8	13.4	(below)
7 y.o.	28.06	27.01	28.73	1.28	1.26	1.30	17.0	17.0	
8 y.o.	30.52	30.06	31.02	1.34	1.33	1.34	17.0	17.3	
9 y.o.	37.73	34.78	40.68	1.43	1.42	1.44	17.2	19.6	(above)
10 y.o.	39.73	37.56	41.70	1.49	1.48	1.49	17.1	18.8	(normal)
Total	21.76	22.68	20.31	1.24	1.24	1.23	–	–	

Legend: BMI – body mass index it was calculated using the BMI Percentile Calculator for Child and Teen from Centers for Disease Control and Prevention of the U.S. Department of Health & Human Services (CDC, 2021).

Regarding the processing of milk, the applied questionnaire in this work was created to assemble data about the favorite milk by Brazilian consumers and, of course, the daily ingestion amount. We asked about the consumption of three types of the product: raw milk, pasteurized milk, or UHT milk. Raw milk is obtained from farmed animals that have not been treated thermically, and it typically contains around 4,4 g of fat per 100 g of milk. As a biological fluid, raw milk is a sterile secretion; however, the contact with udder or other sources as

environmental ones turns it into a very perishable product. Ultra-high temperature (UHT) milk is heated in a continuous process to temperatures higher than 135-140 °C for a few seconds (usually 1-8 sec), followed by instant cooling and aseptic packing into sterile bottles or boxes, resulting in a product shelf- stable for months. Another important aspect of milk intake is the fat content of the product. There is a standardization of milk fat content, which enables consumers to choose the desired fat content. UHT whole milk contains about 3% of fat, while semi-skimmed and skimmed milk has 1.7% and 0.1 to 0.3% of fat (CHAVAN *et al*., 2016; SUGRUE *et al*., 2019).

In this assessment of milk consumption, we observe that the UHT whole-milk consumption by infants of São Carlos city is higher than pasteurized milk or raw milk ingesting. This result corroborates the milk consumption preferences of the Brazilian population once UHT products have a more extended expiration date than pasteurized or raw milk and dismiss refrigeration before opening the packing (SIQUEIRA, 2019). Pasteurized whole milk is the second type most consumed, followed by raw milk consumption. It is important to highlight that raw milk commercialization was banned in 1970, being its ingestion not recommended due to possible risks to human health from microbiologic contaminations (BRASIL, 1970; FDA, 2018). We may also notice that different age ranges similar intake volumes of milk, suggesting that the habit of milk intake prevails along with childhood.

Another important outcome from this complementary study is an assessment of individuals with dietary restrictions to consumption of cow milk. There are two main types of cow milk ingesting restrictions: *(i)* lactose intolerance, which is the incapacity to digest the carbohydrate lactose; and *(ii)* milk protein allergy (also known as cow's milk protein allergy – CMPA), observed when a person presents adverse reactions from immunological origin to cow's milk ingestion (RANGEL *et al*., 2016; MARANGONI *et al*., 2019; VERDUCI *et al*., 2019). In this research, we noted that 7.08% of interviewed children presented a restriction to cow's milk consumption. An increasing or decreasing trend in the number of individuals with milk consumption restriction through different ages considered in this work was not observed. Because the general public easily confounds lactose intolerance and milk protein allergy, there was no specific discrimination about the type of restriction.

Perspectives: The availability of qualitative data from the children's questionnaires about milk consumption might be particularly useful to future research in toxicology, nutrition and other areas; e.g., to estimate daily intake of some contaminant as pesticides, heavy metals, or mycotoxins through milk intake.

ATTACHMENT A

Authorization documents for the "*Pesquisa sobre Hábitos Alimentares para Estimar a Ingestão Diária de Leite*"

PREFEITURA MUNICIPAL DE SÃO CARLOS
Secretaria Municipal de Educação
Rua 13 de naio,2056, Centro – CEP: 13560-647 - São Carlos – SP
Telefone: (16) 3373-3222 / Fax: 3373-3227
E-mail: educacao@saocarlos.sp.gov.br

São Carlos, 02 de maio de 2017

Ilmo Sr.
Orlando Mengatti Filho
Secretário Municipal de Educação

A Divisão de Ensino Fundamental da Secretaria Municipal de Educação analisou o projeto de pesquisa da aluna pós-graduanda Letícia Aparecida Marques, da Universidade de São Paulo, vinculado ao Programa de Pós Graduação em Química, intitulado: **"PESQUISA SOBRE HÁBITOS ALIMENTARES PARA ESTIMAR A INGESTÃO DIÁRIA DE LEITE"** sob a orientação do professor Drº Stanislau Bougusz Junior.

O trabalho tem por objetivo verificar o consumo de diário de leite pelas crianças em idade entre 6 e 10 anos da cidade de São Carlos. Para levantamento dos dados será aplicado um questionário que as crianças levarão para casa para preenchimento pelos responsáveis, no dia marcado para a coleta dos questionários será feita a pesagem das crianças para e medição da altura para possíveis constatações.

Considerando a importância de pesquisa nesta área para o avanço do conhecimento para possíveis atuações e apontamentos, a Divisão de Ensino Fundamental considerou procedente o pedido de autorização desde que haja as devidas autorizações dos participantes, os dados de pesquisa sejam de uso exclusivo para fins acadêmicos, não sendo permitido o uso de imagem dos alunos, professores e equipe escolar.

Pede-se, por gentileza, que a pesquisadora planeje previamente os dias e horários da coleta de dados de modo a não comprometer a rotina diária da escola e também se comprometa a trazer uma devolutiva no final do trabalho realizado a esta Secretaria. A pesquisa só poderá iniciar após o parecer positivo da comitê de ética da instituição.

Atenciosamente,

Profª Cilmara Aparecida Seneme Ruy
Diretora de Departamento Pedagógico – SME / São Carlos

UFSCAR - UNIVERSIDADE FEDERAL DE SÃO CARLOS

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Pesquisa sobre Hábitos Alimentares para estimar a Ingestão Diária de Leite
Pesquisador: Letícia Aparecida Marques
Área Temática:
Versão: 3
CAAE: 65685317.9.0000.5504
Instituição Proponente: UNIVERSIDADE DE SAO PAULO
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.260.550

Apresentação do Projeto:
Trata-se de um estudo de caráter tipo transversal, quantitativo com aplicação de questionário elaborado para estimativa de consumo de leite por crianças em idade escolar (6 a 10 anos) da cidade de São Carlos - SP, e realização de medidas de peso e altura corporal dos participantes voluntários.
O hábito do consumo de leite desde a infância faz com que a exposição não ocupacional dos seres humanos a resíduos de pesticidas organoclorados através do leite seja uma preocupação constante da Organização Mundial da Saúde e do Ministério da Agricultura, Pecuária e
Abastecimento. Pesquisas recentes a este respeito tem realizado o monitoramento destes compostos em produtos de origem animal em paralelo a pesquisa sobre hábitos alimentares a fim de estimar quanto destes pesticidas estão sendo ingeridos pelo consumo destes alimentos. No Brasil, poucos são os grupos de pesquisa que se ocupam de tal investigação. Em função disso, foi elaborado o presente projeto de pesquisa

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que tem por
objetivos: i) estimar a ingestão diária de leite por crianças em idade escolar para correlacionar aos níveis de contaminação por resíduos de compostos organoclorados (PCBs). Esta estratégia fornecerá valiosas informações sobre os níveis de contaminação do leite integral produzido no estado de São Paulo, bem como estimará a ingestão diária destes compostos por crianças em idade escolar o que fornecerá subsídios importantes para políticas públicas de aumento da qualidade de alimentos no que diz respeito à presença de resíduos de organoclorados (PCBs) em leite

Objetivo da Pesquisa:
Estimar a ingestão diária de leite integral por crianças de 6 a 10 anos aplicando Questionário sobre Hábitos Alimentares em Escolas Municipais da
cidade de São Carlos – SP.

Avaliação dos Riscos e Benefícios:
Riscos:
A pesquisa envolve riscos mínimos. É possível que a criança sinta-se desconfortável ou envergonhada ao responder o Questionário sobre Consumo Alimentar. Este questionário será totalmente anônimo e a criança participante irá respondê-lo em casa, junto aos pais, evitando possíveis dúvidas e constrangimentos decorrentes do preenchimento em sala de aula. As medidas de altura e peso das crianças participantes serão efetuadas individualmente, fora da sala de aula, também de modo a evitar constrangimentos. Todos dados coletados serão mantidos sob sigilo e utilizados apenas para os fins desta pesquisa.
Benefícios:
Estimando a ingestão diária de PBCs em leite por crianças será possível fornecer importantes informações para os órgãos fiscalizadores nacionais (como Agência Nacional de Vigilância Sanitária - ANVISA,

Ministério
da Agricultura, Pecuária e Abastecimento - MAPA), colaborando com estes para a verificação e adequação das normativas e legislações brasileiras em relação aos níveis reais de exposição.
- Os riscos e benefícios foram descritos pelo pesquisador (a) e pode-se aferir que os benefícios indiretos citados suplantam os riscos de participação na pesquisa.

Comentários e Considerações sobre a Pesquisa:

- Pesquisa bem estruturada e fundamentada cientificamente, importante para a área do estudo.

Considerações sobre os Termos de apresentação obrigatória:

- Foram anexados os seguintes documentos obrigatórios: folha de rosto assinada,TCLE/TALE do participante, projeto de pesquisa e Informações básicas e autorização do órgão responsável.
-A folha de rosto confere com o título do projeto de pesquisa e apresenta a assinatura do pesquisador e do responsável pela instituição conforme a resolução 466/12 do CNS/MS.

Recomendações:

Sem novas recomendações.

Conclusões ou Pendências e Lista de Inadequações:

Todas as pendência apontadas nos pareceres anteriores foram corrigidas.

Considerações Finais a critério do CEP:

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_856057.pdf	10/07/2017 11:03:42		Aceito
Projeto Detalhado / Brochura Investigador	PlataformaBrasil_Projeto_CEP_Pesquisa_ingestao_diaria_leite_1007.pdf	10/07/2017 11:02:42	Letícia Aparecida Marques	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	PlataformaBrasil_TERMO_DE_ASSENTIMENTO_6a10anos_corrigido_1007.docx	10/07/2017 11:02:22	Letícia Aparecida Marques	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	PlataformaBrasil_TCLE_corrigido_1007.docx	10/07/2017 11:02:05	Letícia Aparecida Marques	Aceito

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Continuação do Parecer: 2.260.550

Outros	Autorizacao_Secretaria_Municipal_Educacao_Sao_Carlos.pdf	09/05/2017 14:22:08	Letícia Aparecida Marques	Aceito
Folha de Rosto	Folha_de_rosto_0103.pdf	01/03/2017 16:29:51	Letícia Aparecida Marques	Aceito
Outros	Questionario_Consumo_Alimentar_v2002.docx	20/02/2017 14:31:52	Letícia Aparecida Marques	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

SAO CARLOS, 05 de Setembro de 2017

Assinado por:
Priscilla Hortense
(Coordenador)

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