الجمهورية الجزائرية الديمقراطية الشعبية

People's Democratic Republic of Algeria

وزارة التعليم العالي والبحث العلمي

Ministry of Higher Education and Scientific Research

جامعة8ماي1945قالمة

University of Guelma – 8 May 1945

Faculty of Natural and Life Sciences and Earth and Universe Sciences

Thesis In View of Obtaining the Master's Degree

Domain: Natural and Life Sciences

Sector: Biological Sciences

Specialty / Option: APPLIED BIOCHEMISTRY

Department of Biology

Study of the subacute toxicity of an association of two endocrine disruptors triclosan and bisphenol A, in female Wistar rats

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June 2024

Acknowledgment

Praise be to **ALLAH**, whose grace has guided us through this journey, granting us success, patience, and, above all, good health.

I would like to extend my sincere gratitude to the members of my thesis committee, whose contributions have been pivotal in the completion of this research.

Firstly, I would like to thank the jury president, **Pr. Benouareth Djamel-Eddine** for their valuable time and for overseeing this process with dedication and professionalism. Your insightful comments and thoughtful questions have greatly enriched this work.

I am deeply grateful to my examiner, **Dr.Boussenane Nadia**, for their meticulous review and constructive feedback. Your expertise and critical evaluation have been instrumental in refining and improving the quality of this thesis.

To our beloved supervisor, **Miss. Merabet Rym**, whose encouragement, valuable insights, and unwavering support have been instrumental in our journey. Her thoughtful guidance has made a profound impact on our work. Words seem inadequate to express the depth of our appreciation for your unwavering support and guidance. Your belief in us, your gentle encouragement, and your endless well of wisdom have been like a beacon of light, guiding us through even the darkest moments. Your kindness has touched our hearts in ways we cannot fully articulate, and for that, we are eternally grateful.

We also extend our sincere appreciation to our co-supervisor, **Dr. Benosmane Sana**, whose generosity, patience, and support have been invaluable throughout our research. Their guidance has illuminated our path toward excellence.

We express our gratitude to the team of the Wastewater Treatment Plant (WWTP) for their invaluable cooperation in providing us with the necessary water quantities crucial to our graduation project.

We extend our heartfelt appreciation to **Mr. Mahdi, Mrs. Hanane**, and **Mrs. Ghania** for ensuring suitable working conditions within the animal house, and to **Mrs. Nassima** in the laboratory, whose assistance greatly facilitated our project.

Furthermore, we extend our sincere thanks to the hospital **Institution ibn Zohr Guelma**, particularly the Anatomy section, represented by **Dr. Azouzi** and **Mrs. Fatiha**, for their cooperation and assistance in the applied part of our work.

A special thank you to **Khrakheria Selma**, **Guelmi Ines**, and **Chabani Amani Nore al Islam** for their unconditional support and guidance throughout this project.Their expertise, availability, and patience were major assets in carrying out this research. Their enlightened advice and their confidence allowed us to progress and achieve results of which we are proud.

In conclusion, we extend our heartfelt gratitude to all individuals, both near and far, whose contributions have shaped the development of this dissertation. We express our deepest appreciation to our beloved parents, whose unwavering support and encouragement have been a constant source of inspiration throughout our journey. We are also grateful to all members of the **BA class of 2023-2024** for their camaraderie and shared experiences.

Dedication

*First and foremost, I thank **"ALLH"** who illuminated my path and granted me the strength and perseverance necessary to accomplish this work.*

*To my dear mother **"Zohra"** for your love and affection that envelop me, your kindness that guides me, and your presence by my side which has always been my source of strength to face various obstacles.*

*To my beloved father **" Kamel "** who instilled respect and love for science in me. Thanks to you, Dad, I have learned the value of work and responsibility. You are the best father who has always done his best for us.*

*And to my brothers **Abderraouf** and **Taqiy Eddin**, for their constant support and encouragement throughout my university journey.*

*My exceptional supervisor, **Dr. Merabet Rym** Your unwavering guidance, support, and encouragement have been the cornerstone of my success. Your wisdom and patience have not only shaped my professional journey but also enriched my personal growth. I am profoundly grateful for your belief in my potential, your constructive feedback, and your inspiring leadership. Thank you for being a constant source of motivation*

*To my sister from another mother, **Nardjes**, for your precious friendship and unconditional love. Your support and inspiring encouragement made this journey much sweeter. Thank you for always supporting me in my decisions.*

*To my best friend **Panda**, I am deeply grateful for your presence and your support throughout my academic journey and in life. Your unwavering support has truly made all the difference. Thank you from the bottom of my heart.*

*To my dear friends **Hayem, Ranya, Khaoula**, and **Youssra,** I would thank you from the bottom of my heart for your support and encouragement throughout these years.*

*To my partner, **Zayneb**, who shared precious moments of learning, laughter, and support with me. This thesis results from our collective efforts, and I am grateful to you.*

To everyone who shared this path with me, prayed for me from near or far wished me well, and gave me his hand in moments of my weakness, Thank you all.

With all my love and gratitude,

Chemmakh Imane

Dedication

*Thank **Allah**, who gave me the health and strength to complete this work.*

I dedicate this work

To Myself

*To My dear father **Morad** and my beloved mother **Assia**. You are the main supporters in my journey towards achieving my dreams and completing my studies under the best conditions. I want to extend my deepest thanks to you, a word of encouragement and every moment of support from you was a turning point in my career. I will never forget your sacrifices and your great efforts. I will be grateful to you both for life. You are the reason why I came to this success thank you with all my heart.*

*To my beloved grandmother **Akila**, with a heart full of tenderness and gratitude, I offer you thanks and love. May your heart always remain full of happiness and health.*

*To my dear sisters **Sarah** and **Salsabil**, and my younger brother **Mohammed Taha Abd EL Rahman**, thank you for being by my side and for your endless support and your constant confidence in my abilities.*

*To my supporters in this life, I express my gratitude to you**, Bilal,** for being my source of encouragement, thank you for your unwavering support, and your faith in me. I am eternally grateful for your presence and steadfastness.*

*To all my family **Bensouilah** and **Touami***

***To Dr. Merabet Rym** thank you for all the efforts put into completing this work. I can only express my deep gratitude to your noble spirit, and your endless and unceasing support. You've been more than just a supervisor; you've been like a friend and a sister to me. I feel fortunate to have met a gem like you.*

*To my partner **Imane**, I extend my gratitude for your exceptional dedication in pursuit of our shared objectives. Our collaborative journey has been marked by both triumphs and trials, yet through our joint efforts, we have accomplished much. You are an unparalleled partner.*

*To my dear friends who shared my joys and challenges, **Rania**, **Youssra**, **Khawla**, **Hayam**, and **Meryam**, for always being there for me.*

finally, thanks to everyone who supported me, both near and far.

With love and gratitude, Thank you all.

Bensouileh Zeyneb

List of abbreviations

AR	Androgenic
AOPP	Advance Oxidative Proteins Products
BPA	Bisphenol A
BSA	Bovine Serum Albumin
CYP	Cytochrome P450
DCDD	Dichlorodibenzo-p-dioxin
DDT	Dichlorodiphenyltrichloroethane
DEHP	Di-(2ethylhexyl) phthalate
DES	Diethylstilbestrol
DSP	Daily Sperm Production
DTNB	Dithio-2-nitrobenzoic acid
EDCs	Endocrine-Disrupting Chemicals
ER	Estrogen Receptor
EPA	Environmental Protection Agency
E2	Estradiol
FDA	Food and Drug Administration
H2O2	Hydrogen Peroxide
H&E	Hematoxylin-Eosin
GSH	Glutathione
GSH-Px	Glutathione Peroxidase
LH	Luteinizing Hormone
mTCS	Triclosan-methyl

PBBs Polybrominated Biphenyls

PCBs Polychlorinated Biphenyls

PCOS PolycysticOvarySyndrome

POPs Persistent OrganochlorinePollutants

ROS Reactive Oxygen Species

SD Sprague Dawley

SULT Sulphotransfera

TBA ThiobarbituricAcid

TCA Trichloroacetic Acid

TCDD Tetrachlorodibenzo-p-dioxin

TCS Triclosan

TT Total Testosterone

T4 Thyroxine

TSH Thyroid-Stimulating Hormone

UGT Uridine 5′diphospho-glucuronosyltransferase

MDA Malondialdehyde

WWTP Wastewater Treatment Plant

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Abstract

The present study investigates the subacute toxicity of combined exposure to Triclosan and Bisphenol A in female Wistar rats. Both chemicals are well-known endocrine disruptors, and their ubiquitous presence in consumer products raises concerns about their health impacts. This research aimed to assess the synergistic effects of triclosan and Bisphenol A on oxidative stress in rats. Animals were administered varying doses of the disruptors via subcutaneous and intraperitoneal injections respectively, both individually and in combination, for 25 days. The lowest and highest doses of Bisphenolwere 50 µg/kg/day and 100 mg/kg/day and those of triclosan were 1 mg/kg/day and 10mg/kg/day. Another group was allowed to drink *ad libitum* filtered water collected from Guelma WWTP in order to assess the effectiveness of the treatment methods of that station. Various parameters were evaluated to understand the potential adverse consequences. The study assessed oxidative stress biomarkers and histopathological changes in the liver, kidneys, and ovaries. Our finding indicated significant alterations in relative organ weights, suggesting systemic stress in all treated animals. Results demonstrated significant physiological alterations in the organs of animals after exposure to a high dose combination of the two chemicals and the high dose of Bisphenol A leading to disruptions in oxidative balance. Elevated levels of AOPPs and MDA confirmed the presence of oxidative stress.There was a notable decrease in serum glucose levels in groups treated with low-dose mixture, indicating metabolic disruption. These changes were accompanied by discernible hepatic and ovarian damage, confirmed through histopathological analysis. The findings underscore the potential health risks posed by exposure to these chemicals and advocate for heightened regulatory scrutiny and further research to mitigate human exposure to those chemicals.

Keywords: endocrine disruptor, bisphenol A, triclosan, subchronic toxicity, oxidative stress, liver, kidney, ovary.

Résumé

La présente étude explore la toxicité subaiguë d'une exposition combinée au triclosan et au bisphénol A chez les rates Wistar. Les deux substances chimiques sont bien connues comme perturbateurs endocriniens, et leur présence ubiquitaire dans les produits de consommation suscite des inquiétudes quant à leurs impacts sur la santé. Cette recherche visait à évaluer les effets synergiques du triclosan et duBisphénol A sur le stress oxydatif chez les rats. Les animaux ont reçu diverses doses de ces perturbateurs par injections sous-cutanées et intrapéritonéales respectivement, à la fois individuellement et en combinaison, pendant 25 jours. Les doses les plus faibles et les plus élevées de bisphénol étaient de 50 µg/kg/jour et 100 mg/kg/jour, et celles de Triclosan étaient de 1 mg/kg/jour et 10 mg/kg/jour. Un autre groupe a été autorisé à boire à volonté de l'eau filtrée collectée de la station d'épuration de Guelma afin d'évaluer l'efficacité des méthodes de traitement de cette station. Divers paramètres ont été évalués pour comprendre les éventuelles conséquences néfastes. L'étude a évalué les biomarqueurs de stress oxydatif et les modifications histopathologiques dans le foie, les reins et les ovaires. Nos résultats ont révélé des altérations significatives des poids relatifs des organes, suggérant un stress systémique chez tous les animaux traités. Les résultats ont montré des altérations physiologiques significatives dans les organes des animaux après exposition à une combinaison à dose élevée des deux produits chimiques et à la dose élevée du bisphénol A, entraînant des perturbations de l'équilibre oxydatif. Des niveaux élevés d'AOPP et de MDA ont confirmé la présence de stress oxydatif. Une diminution notable des niveaux de glucose sérique dans les groupes traités avec le mélange à faible dose a indiqué une perturbation métabolique. Ces changements ont été accompagnés de dommages hépatiques et ovariens discernables, confirmés par analyse histopathologique. Les résultats soulignent les risques potentiels pour la santé posés par l'exposition à ces substances chimiques et plaident pour une surveillance réglementaire accrue et des recherches supplémentaires afin de réduire l'exposition humaine à ces polluants.

Mots-clés : perturbateur endocrinien, bisphénol A, triclosan, toxicité subchronique, stress oxydatif, foie, rein, ovaire.

الملخص

تبحث هذه الدراسة في السمية تحت الحادة للتعرض المشترك للتريكلوسان والبيسفينول أ في إناث الجرذان من سلالة ويستار. كلا المادتين الكيميائيتين معروفتان جيدًا كمعطلات الغدد الصماء، وحضورهم الواسع في المنتجات الاستهلاكية يثير مخاوف حول تأثيراتهم الصحية. هدفت هذه الدراسة إلى تقييم التأثيرات التآزرية للتريكلوسان والبيسفينول أ على الإجهاد التأكسدي في الجرذان. تم إعطاء الحيوانات جرعات مختلفة من التريكلوسان والبيسفينول أ عن طريق الحقن تحت الجلد والحقن داخل الصفاق على التوالي، بشكل فردي ومشترك، لمدة 25 يومًا. كانت أدنى وأعلى جرعات البيسفينول أ هي 50 ميكروغرام/كغ/يوم و100 ملغ/كغ/يوم، بينما كانت جرعات التريكلوسان 1 ملغ/كغ/يوم و10 ملغ/كغ/يوم. مجموعة أخرى سُمِح لها بشرب الماء المفلتر بحرية والذي تم جمعه من محطة معالجة مياه الصرف الصحي في قالمة لتقييم فعالية أساليب المعالجة في تلك المحطة. تم تقييم العديد من المعايير لفهم العواقب الضارة المحتملة. قامت الدراسة بتقييم مؤشرات الإجهاد التأكسدي والتغيرات النسيجية المرضية في الكبد والكلى والمبيضين. أشارت نتائجنا إلى تغيرات كبيرة في الأوزان النسبية للأعضاء، مما يشير إلى وجود إجهاد نظامي في جميع الحيوانات المعالجة. أظهرت النتائج تغيرات فسيولوجية كبيرة في أعضاء الحيوانات بعد التعرض لجرعة عالية من مزيج المواد الكيميائية وجرعة عالية من البيسفينول أ، مما أدى إلى اضطرابات في التوازن التأكسدي. أكدت المستويات المرتفعة منAOPPsو MDAوجود الإجهاد التأكسدي. لوحظ انخفاض ملحوظ في مستويات الجلوكوز في الدم في المجموعات المعالجة بالمزيج منخفض الجرعة، مما يشير إلى اضطراب في التمثيل الغذائي. رافق هذه التغيرات أضرار واضحة في الكبد والمبيضين، تم تأكيدها من خلال التحليل النسيجي المرضي. تؤكد النتائج على المخاطر الصحية المحتملة الناجمة عن التعرض لهذه المواد الكيميائية وتدعو إلى زيادة التدقيق التنظيمي وإجراء المزيد من الأبحاث للتخفيف من تعرض البشر للتريكلوسان والبيسفينول أ .

الكلمات المفتاحية: معطل الغدد الصماء، بيسفينول أ، تريكلوسان، السمية تحت المزمنة، الإجهاد التأكسدي، الكبد، الكلى، المبيض.

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Introduction

Increased global industrialization has exposed humans to a variety of industrial chemicals which can interfere with the endocrine system. As such, they have been defined as endocrine disruptors, also known as endocrine-disrupting chemicals (EDCs) **(Srnovršnik et al., 2023).**

EDCs are a group of hormonally active agents to which we are directly or indirectly exposed daily and that have been widely used in industry for almost a century.They are considered substances that interfere with the natural hormones responsible for maintaining homeostasis, reproduction, development, and behavior by affecting their synthesis, secretion, transportation, binding, or elimination in the body **(Leonardi et al., 2017)**. Their interference with the hormonal system can negatively impact the development, reproduction, neurological function, cardiovascular health, immune system, and metabolism in both humans and wildlife. These effects may become evident after prolonged exposure and can also affect future generations **(Presunto et al., 2023).**

Bisphenol A (BPA) and triclosan (TCS), are two mass-produced and widespread EDCs **(Yuan et al., 2015)**. The European Chemicals Agency has recently designated BPA as a substance of very high concern and a recognized EDC that can alter hormonal balance even at low levels **(Mustieles et al., 2020)**. This crystalline chemical compound is widely used as a key monomer of epoxy resins and polycarbonate (PC) plastics for more than 50 years **(Castellini et al., 2020)**.It is a major high-production chemical, with over 6 billion pounds produced each year and over 100 tons are estimated to be released into the atmosphere **(Inadera, 2015).**

Currently, the detection rate of TCS in the environment is quite high and almost ubiquitous. TCS is a widespread environmental contaminant and has been determined in urine, blood, breast milk, amniotic fluid, and so on from the general population in different parts of the world**(Chen et al., 2023).**Triclosan is a broad-spectrum, lipophilic, antibacterial agent, developed in the 1960s**(Alfhili and Lee, 2019)**. As a polychlorinated bisphenol compound, TCS has been extensively used in a variety of consumer products **(Weatherly and Gosse, 2017),**such as toothpaste, hand soaps, shampoos, cosmetics, and other consumer products, as well as in clinical settings (antiseptics, disinfectants) and medical devicanes**(Montagnini et al., 2021).**

Recent research has focused on understanding the synergistic effects of BPA and TCS when combined, as humans are frequently exposed to multiple chemicals simultaneously rather than in isolation. The endocrine system, being highly sensitive to hormonal balance,

can be disrupted by these chemicals, potentially leading to adverse health outcomes such as reproductive disorders, developmental issues, metabolic changes, and even cancer. Investigating the combined impact of bisphenol A and triclosan is critical for developing comprehensive risk assessments and informing regulatory policies to protect public health **(Cimmino et al., 2020).**

This study aims to explore both individual and joint effects of a daily exposure of female Wistar rats to TCS and BPA in inducing oxidative stress and altering endocrine systems. It also highlights the necessity for more stringent control measures to mitigate the risks associated with these pervasive endocrine disruptors.

In this master dissertation, the research is structured into several key sections. The first section introduces the topic, including a literature review of endocrine disruptors, especially BPA and TCS, their toxicity effect and impact, The endocrine system, and its disruptors. The second section presents the methodology employed to collect and analyze data, outlining the experimental design and data collection procedures. The third section presents the results obtained from the analysis, along with their interpretation and discussion.Finally, the conclusion summarizes the key findings, discusses their significance, and suggestsavenues for future research.

Literature review

Chapter I
The Endocrine System and Its Disruptors

I.Endocrine system

The endocrine system, a complex network of glands distributed throughout the body, is crucial in maintaining homeostasis and governing vital functions like reproduction and development, including regulating sex hormones**(Quignot et al., 2012).**From conception through adulthood, this system oversees various biological processes such as general growth, brain and nervous system development, reproductive system function, metabolism, and blood sugar levels. Comprising glands like the hypothalamus, pituitary, and thyroid, as well as organs like ovaries and testes, shown in (**Figure 1**) the human endocrine system meticulously produces and releases different types of hormones to perform diverse functions**(Khetan, 2014).** Acting through the bloodstream, these hormones exert longer-lasting effects on nearly every organ and cell, influencing metabolism, energy levels, electrolyte balance, growth, development, and reproduction **(Rachdaoui and Sarkar, 2013).**

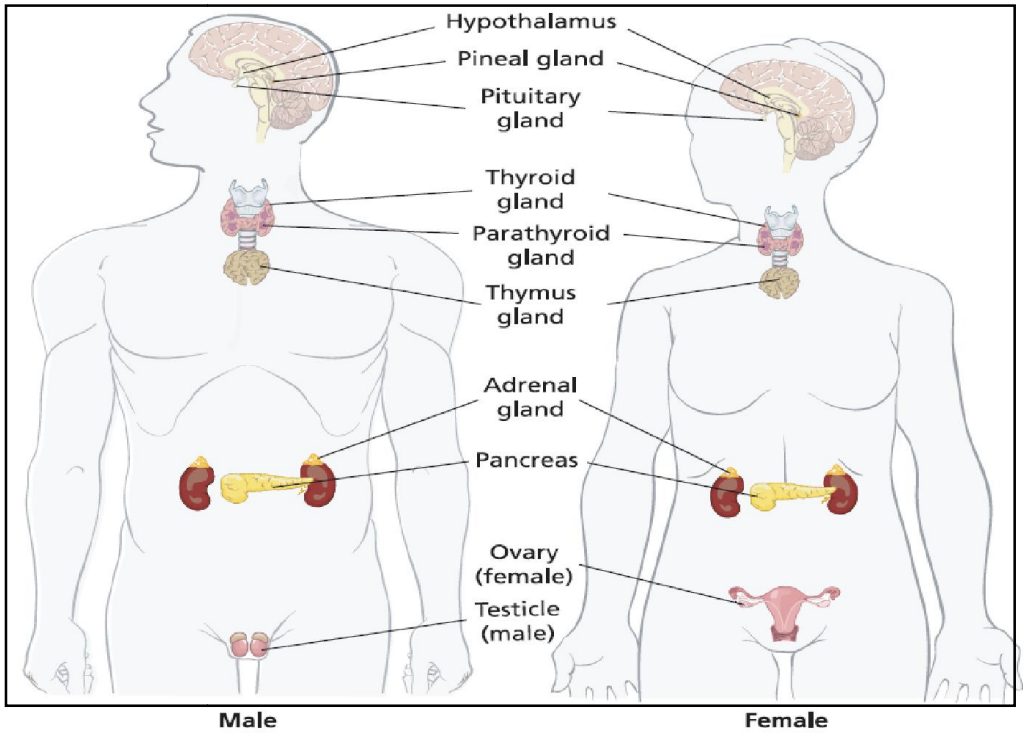

Figure 1: Endocrine system **(Rushton, 2009).**

I.1. Hormones

Hormones are molecules created by endocrine glands such as the hypothalamus, pituitary gland, adrenal glands, gonads, thyroid gland, parathyroid glands, and pancreas. They are released into the bloodstream in response to certain stimuli and travel to their target cells where they interact with receptors either on or inside the cell. This triggers

biochemical reactions that modify the cell's function or activity**(Hiller-Sturmhöfel and Bartke, 1998).**

Hormones are transported through the blood and may require specific vehicles, although most move freely through circulation and can be susceptible to enzymatic degradation. Through coordinated release mechanisms involving multiple cells, hormones reach target organs in sufficient concentrations **(Bernhard Kleine and Winfried G. Rossmanith, 2016).**Despite circulating throughout the body, hormones affect only specific cells— their target cells— due to the presence of receptors. Each cell type possesses a unique set of receptor proteins, facilitating hormone binding and subsequent cellular responses **(Rushton, 2009).**

I.2. Classes of hormones

Hormones can be categorized into three classes according to their major components: peptide hormones, amino acid analogs, and steroid hormones.

I.2.1. Peptide hormones

Are highly diverse and abundant in nature, characterized by a wide array of sizes, compositions, chain numbers, modifications, and production mechanisms. Examples range from large single-chain peptides like the 192-amino acid growth hormone (GH) to small cyclic peptides like TRH composed of just three amino acids, along with prolactin. Notably, anterior pituitary hormones like thyrotropin (TSH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) are glycosylated and consist of two chains each, sharing a common α chain while possessing a distinct β chain that imparts specificity to the hormone **(Leung and Farwell, 2018)**.

I.2.2. Amino acid hormones

Are water-soluble compounds derived from amino acids specifically, the amine-based hormones are derived from tyrosine and are secreted by the thyroid (known as iodothyronines) and the adrenal medulla.

Thyroglobulin, a large glycoprotein containing over 100 tyrosine residues, serves as the precursor for iodothyronines and is synthesized by thyroid follicular cells. Iodothyronines are formed through the iodination and coupling of two tyrosines and are the only iodinated compounds with significant biological activity. In adrenal catecholamine-secreting cells, tyrosine is sequentially converted into dopamine, norepinephrine, and epinephrine.

Serotonin, also called 5-hydroxytryptamine, is derived from tryptophan **(Leung and Farwell, 2018).**

I.2.3. The steroid hormones

The body produces steroid hormones in the adrenal cortex, gonads, and placenta, all of which are derived from cholesterol and have significant clinical importance. Mitochondria and smooth endoplasmic reticulum are responsible for steroid hormone synthesis, and since they are lipophilic, they cannot be stored in vesicles and are synthesized on demand as precursors. When the parent cell is stimulated, steroid hormone precursors are converted into active hormones, and as their intracellular concentration increases, they diffuse out of the parent cell via simple diffusion. As steroids are derived from cholesterol, they are not soluble in plasma and other body fluids, so they bind to transport proteins that increase their half-life and ensure ubiquitous distribution. The equilibrium of protein-bound steroids and a small fraction of free steroids, which are 'active,' ensures their proper functioning. Steroids can work quickly by binding to cell surface receptors or slowly by binding to cytoplasmic or nucleic receptors and ultimately activate gene transcription. Cortisol, 11-deoxycortisol, aldosterone, corticosterone, and 11-deoxycorticosterone are the steroids primarily produced in the adrenal glands, while most other steroid hormones, including estrogens, are produced in both the adrenal glands and gonads **(Holst et al., 2004).**

I.3. Mechanisms of action of hormone

The structural differences affect their mechanisms of action, including whether they can enter their target cells and how they modulate the activity of those cells. The gonads and adrenal cortex produce steroids, which have a molecular structure like that of cholesterol. The molecules can enter their target cells and interact with receptors in the cytoplasm or the cell nucleus. The interaction between hormone-receptor complexes and certain regions of the cell's genetic material (DNA) leads to the regulation of specific hormone-responsive genes.Amino acids hormones are produced by the thyroid gland and another area of the adrenal glands known as the adrenal medulla. Similar to steroids, amino acid derivatives can penetrate cells and bind with receptor proteins that are already linked to specific DNA segments. This interaction leads to changes in the activity of the targeted genes.

Polypeptide and protein hormones are primarily present in the hypothalamus, pituitary gland, and pancreas. Sometimes, they are produced as inactive precursors, called

pro-hormones, which can be cleaved into one or more active hormones. Due to their chemical composition, polypeptide and protein hormones cannot penetrate cells. Instead, they interact with receptors located on the cell surface. This interaction triggers biochemical changes within the cell membrane or interior, ultimately leading to modifications in the cell's activity or function **(Hiller-Sturmhöfel and Bartke, 1998).**

I.4. Roles of hormones

Hormones have many roles that work together to achieve the exquisite regulation required of many body processes. This regulation by the endocrine system is often the result of different mechanisms at many targets, thereby allowing the body to respond to a diverse variety of concurrent physiologic changes and pathologic insults. The major body processes regulated by hormones include energy production, utilization and storage (intermediary metabolism), growth, development, reproduction, and maintenance of the internal environment (mineral and water metabolism and cardiovascular effects)**(Leung and Farwell, 2018)**.

II. Endocrine-disrupting chemicals

II.1. Definition

An endocrine-disrupting compound as defined by the U.S. Environmental Protection Agency (EPA)isan exogenous agent that interferes with the body's natural hormonal processes, critical for maintaining balance, reproduction, and development **(Diamanti-Kandarakis et al., 2009).**These compounds, whether natural or synthetic, can disrupt the synthesis, secretion, transport,metabolism, binding action, or elimination of blood-borne hormones.Over time, EDCs accumulate in the environment and enter the human body through various pathways, including water, air, food, and everyday objects, with the potential to transfer from mother to fetus or baby via placenta or breast milk **(Özen and Darcan, 2011).**

Exposure to EDCs can disrupt hormonal signaling pathways resulting in a wide range of adverse effects on reproduction, growth, behavior, and overall health, including obesity, diseases, and even epigenetic changes affecting future generations**(Cooke et al., 2013).** These compounds have the potential to interfere with the normal functioning of the endocrine system by targeting the glands and hormones as shown in (**Figure 2**) that regulate critical bodily processes, such as metabolic rate, sexual development, insulin production and utilization, stress response, and gender behavior **(Preda et al., 2012).** Such

disruptions can have profound consequences on individual health and may extend to impact subsequent generations.

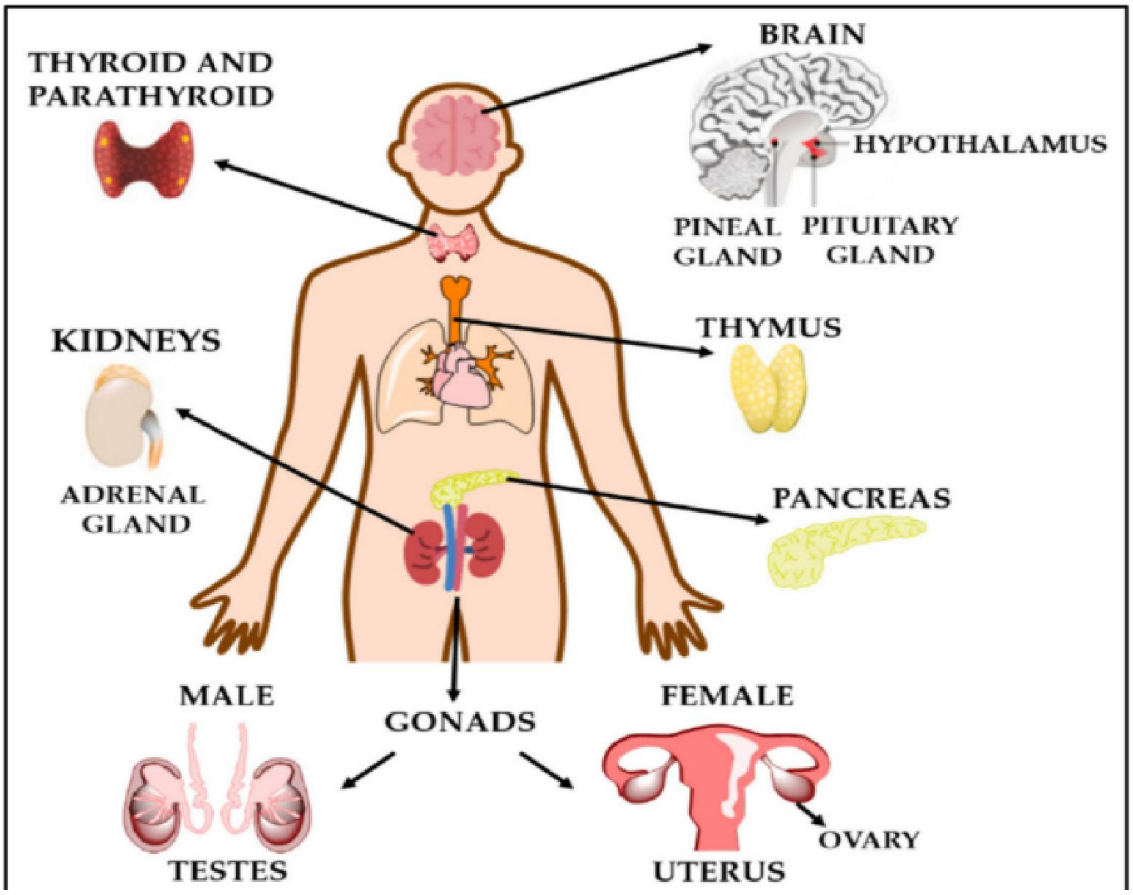

Figure 2: Representation of main endocrine glands targeted by EDCs **(Pironti et al., 2021)**.

II.2. Classification of EDCs

The group of molecules identified as endocrine disruptors is highly heterogeneous and includes:

II.2.1. Natural endocrine disruptors

The best-known chemicals in this group are phytoestrogens, which are relatively weak compared to endogenous estrogen. They are found in several nutrients frequently consumed daily (i.e., carrots, garlic, apple, coffee, cherry, parsley, legumes). Phytoestrogens have estrogenic effects when consumed in huge amounts and antiestrogenic effects at low concentrations **(Özen and Darcan, 2011)**.

II.2.2. Synthetic endocrine disruptors

The category of molecules identified as endocrine disruptors presents a diverse range, including synthetic chemicals employed as industrial solvents/lubricants and their resultant byproducts like polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), and dioxins. Additionally, plastics such as bisphenol A (BPA), plasticizers like phthalates, pesticides including methoxychlor, chlorpyrifos, dichlorodiphenyltrichloroethane (DDT), fungicides like vinclozolin, and pharmaceutical agents like diethylstilbestrol (DES) are also implicated **(Diamanti-Kandarakis et al., 2009).**As neutralization or inactivation is difficult and most of these substances often accumulate in fat tissue, they may persist in the body for long periods and cause harmful effects **(Özen and Darcan, 2011)**.

II.3.Routes of exposure to EDCs

Humans are regularly exposed to a wide array of chemicals through various means, often without conscious awareness. Endocrine-disrupting chemicals (EDCs) primarily enter the human body through ingestion, although inhalation and dermal absorption also play a role to some extent. The main pathway for Persistent Organochlorine Pollutants (POPs) and other EDCs to reach humans is through dietary intake, accounting for more than 90% of total chemical exposure. These substances, especially POPs, are known for their lipophilic nature, meaning they have an affinity for fats and can accumulate in fatty tissues within the body.

Once released into the environment, these chemicals can contaminate food sources such as fish, meat, dairy, and poultry, ultimately reaching humans through ingestion. POPs can persist in the body's fatty tissue for extended periods, leading to prolonged exposure. Certain environmental chemicals can also become airborne and enter the body through inhalation, particularly volatile and semi-volatile compounds. Inhalation can significantly influence exposure, especially for chemicals that readily evaporate into the air **(Yilmaz et al., 2020)**.

II.4.Mechanisms of action

Endocrine-disrupting chemicals (EDCs) disrupt the body's normal functions by either imitating or obstructing hormones shown in (**Figure 03**). They can cause disruptions through various mechanisms:

- Modifying nuclear receptor signaling, such as estrogen receptors, androgen receptors, progesterone receptors, thyroid receptors, retinoid receptors, and others.
- Influencing nonsteroidal receptors, transcription coactivators, and enzymatic pathways involved in steroid biosynthesis and metabolism.
- Imitating natural hormones, triggering the body to react to the stimulus or respond improperly.
- Blocking hormone effects from specific receptors, and directly or indirectly affecting hormone production levels.

When an EDC binds to hormone receptors, it can activate or hinder the cellular response typical of the replaced hormone. EDCs with diverse structures have been observed to compete for estrogen-binding sites, resulting in varied responses. Some common features of EDCs include resistance to metabolism, accumulation in the body, lipophilicity, and a shared structural basis involving phenols or similar functional groups**(Jones and Regan, 2018)**.

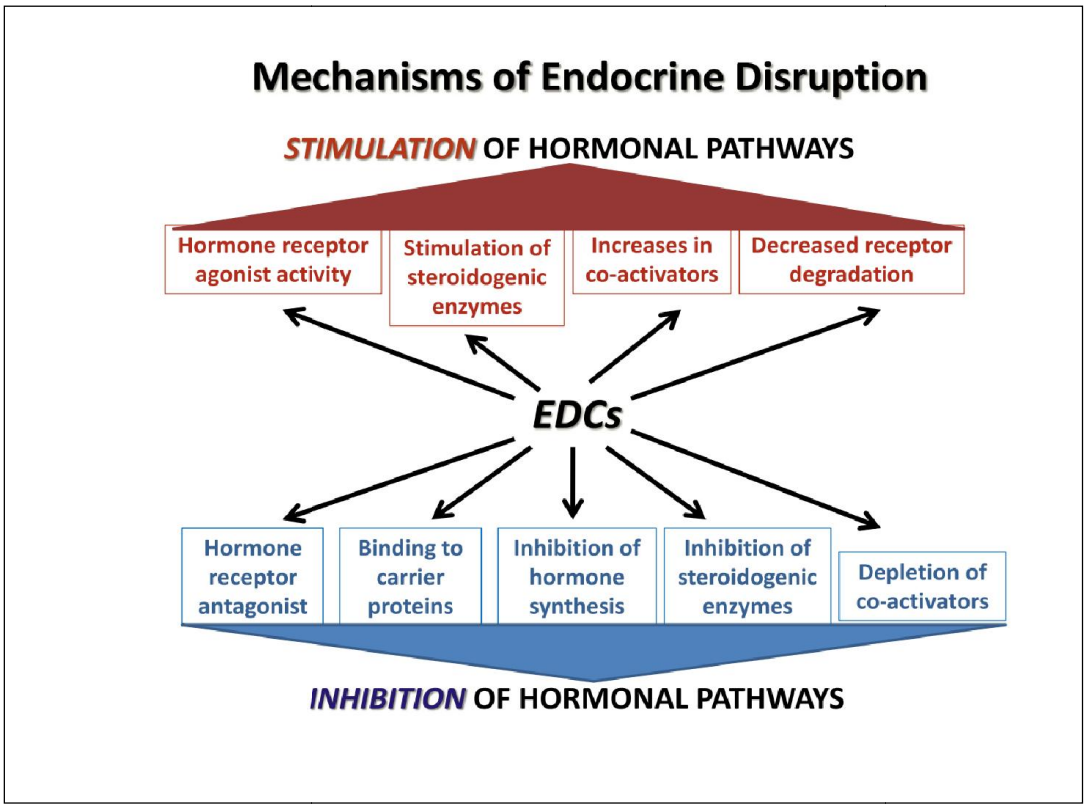

Figure 3: Mechanisms of endocrine disruptions**(Cooke et al., 2013)**.

II.5.Endocrine-disrupting compounds

A wide range of chemicals possess endocrine-disrupting properties, including bisphenol A, organochlorines, polybrominated flame retardants, alkylphenols, and phthalates. These compounds have been identified as risk factors for a variety of diseases, such as reproductive, neural, and metabolic diseases, as well as cancers **(Ahn and Jeung, 2023)**.

II.5.1.Phthalate

Phthalates, widely utilized as plasticizers and softeners in an array of consumer products including food packaging, toys, cosmetics, and medical devices, rank among the most abundant chemicals globally. Their non-covalent binding to materials facilitates easy diffusion into food, water, and air, amplifying exposure risks.

Notably, di-(2ethylhexyl) phthalate (DEHP) exhibits antagonistic effects on thyroid hormone action, as evidenced by in vitro studies, and induces histopathologic changes in the thyroid gland along with elevated liver enzyme levels in rats, culminating in decreased thyroid hormone levels. Despite inconsistencies, studies on healthy adults reveal varying impacts of phthalate exposure on thyroid-stimulating hormone (TSH) and thyroid hormone levels, with TSH levels unaffected or increased, T3 levels unchanged or decreased, and T4 levels exhibiting no consistent pattern post-exposure. Human exposure to phthalates occurs ubiquitously through ingestion, inhalation, and dermal contact, with medical devices and phthalate-containing products posing significant sources of exposure, particularly DEHP **(Babić Leko et al., 2021).**

Biomonitoring typically relies on urinary phthalate metabolite concentrations to assess exposure levels, highlighting the widespread nature of exposure across demographics, including pregnant women and fetuses, due to the ability of phthalates to cross the placenta **(Diamanti-Kandarakis et al., 2009; Di Pietro et al., 2023).**

II.5.2. Pesticides

Pesticides, classified among the endocrine disruptors in the environment, are a significant concern due to their widespread use in agricultural practices worldwide. Annually, approximately 2.5 million tons of pesticides are introduced into the environment. These chemicals, designed to control pests and weeds, play a crucial role in safeguarding crops, especially in tropical regions where crop loss could reach up to 50% without them. However,the extensive use of pesticides has led to serious environmental

consequences, particularly inaquatic ecosystems, posing risks to human health. Pesticides possess properties that enable themto accumulate in the environment, exhibiting lipophilic traits and enduring long half-lives. Consequently, extensive research has been dedicated to understanding their impact on the environment.

Human exposure to pesticides primarily occurs through ingestion of contaminated food or water, although inhalation and skin contact are also possible routes. Foods such as fish, meat, and dairy products, which contain high lipid content, are common sources of pesticide exposure. Additionally, drinking water, indoor and outdoor air, dust, and soil can also harbor these chemicals. Notably, pesticides such as Dichlorodiphenyltrichloroethane (DDT) and its metabolites tend to accumulate in fatty tissue due to their lipophilic nature.
The long-term presence of pesticides in the body can disrupt reproductive capabilities by altering levels of male and female reproductive hormones. This disruption has been linked to adverse reproductive outcomes such as stillbirth, birth defects, spontaneous abortion, and infertility. Consequently, mitigating pesticide exposure and understanding their effects on human health remain critical priorities in environmental and public health efforts **(Interdonato et al., 2023)**.

II.5.3. Triclosan

It’s a chlorinated aromatic compound that contains functional groups like phenol and ethers. It possesses antimicrobial properties that can affect cell membrane integrity and bacterial lipid synthesis, making it a popular ingredient in numerous consumer products. Triclosan exposure usually occurs through oral and dermal absorption. Fortunately, it isn't persistent in the body, has a biological half-life of less than 24 hours, and is primarily excreted in the urine as a sulfate conjugate or glucuronide. Biomonitoring studies have shown that children and pregnant women are among those who may be exposed to this compound. **(Di Pietro et al., 2023)**.

II.5.4.Dioxins

Polychlorinated dibenzo-p-dioxins, also known as dioxins, are chemicals produced by the incomplete combustion of chlorinated waste and when plastics are exposed to hot surfaces. Exposure to dioxins, including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), can occur through products such as plastic plates and glasses, cleaning substances, or paper whitened with chlorine that interacts with hot surfaces **(Preda et al., 2012).**

Dioxins are prevalent in the environment due to particle transmission in the air and accumulation in plants. Additionally, because they are very resistant, they tend to accumulate in soil and aquifers, polluting them. Humans can be exposed to dioxins through food, mainly meat and dairy products. Therefore, the ingestion of contaminated food is the main source of exposure to the dioxin family **(Interdonato et al., 2023)**.Adverse health effects of dioxin exposure in humans may include cardiovascular disease, diabetes, cancer, endometriosis, early menopause, reduced testosterone, and thyroid hormones. These effects are consistent with adverse health outcomes documented in laboratory and wildlife species**(White and Birnbaum, 2009).**

II.5.5.Bisphenol A

BPAis a significant compound within the bisphenol group, commonly utilized in the production of polycarbonate plastics and epoxy resins on a large scale. These materials find extensive applications in various industries, including food and drink packaging, safety equipment, medical devices, and metal coatings. The widespread use of BPA raises concerns about human exposure due to its capacity to interact with membrane and nuclear receptors like androgen, estrogen, and thyroid receptors. This interaction can lead to disruptions in the endocrine system, potentially causing adverse health effects such as tumors, reproductive issues, and impacts that may span multiple generations**(Gonsioroski et al., 2020)**.

Chapter II
Bisphenol A

I.Definition

Bisphenol A, also known as 4,4'-isopropylidene-diphenol or compound 2,2-bis(4-hydroxyphenyl) propane, is a crystalline substance extensively used as a monomer in industrial processes for the manufacture of plastic materials, polyesters, and polyacrylates **(Santiago et al., 2021).**

Bisphenol A was initially synthesized in 1891 by the Russian chemist A. P. Dyanin. This synthesis involved condensing two phenol molecules with one acetone molecule under the catalytic action of a strong acid or an ion exchange resin featuring acidic sulfonyl groups as shown in **(Figure 04)(Mączka et al., 2022).**

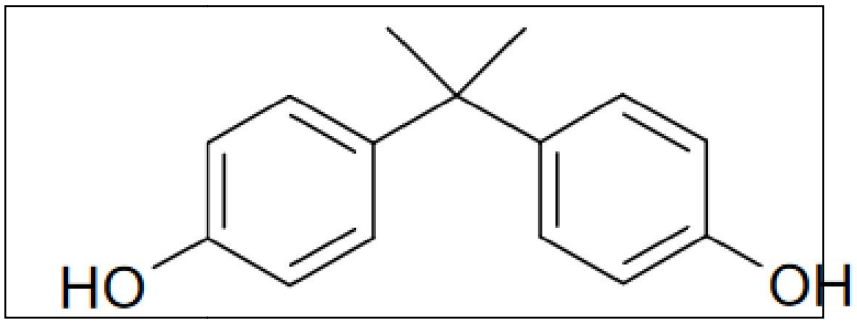

Figure 04: chemical structure of BPA**(Mączka et al., 2022).**

II. Chemical and physical properties

Bisphenol A exists as a white solid with a density of 1.2 g/cm³. Its melting point is 158°C, and it boils at 360°C. It exhibits low solubility in water, ranging from 120 to 300 parts per million at 21.5°C, but it is readily soluble in organic solvents like diethyl ether, acetone, benzene, and ethanol. Additionally, it dissolves well in strong bases and acetic acid **(Mączka et al., 2022).**

The process of synthesizing bisphenols is relatively straightforward. It entails a condensation reaction where phenol reacts with suitable solvents and catalysts. In the case of BPA, two phenol molecules and one molecule of acetone or hydrochloric acid are involved in the synthesis, catalyzed by an ion-exchange resin. For BPF, formaldehyde acts as the solvent,

and a Bronsted acidic ionic liquid serves as the catalyst. On the other hand, BPS is formed by combining phenol with sulfur trioxide. BPF differs from BPA due to the absence of two methyl groups, while BPS features two phenolic functional groups positioned adjacent to the sulfonic group.Consequently, BPF displays lower polarity, while BPS exhibits higher thermal stability **(Mączka et al., 2022).**

III. Applications

Bisphenol A (BPA) is a near-ubiquitous substance in today's world. It's commonly used in food containers, making it nearly unavoidable in household utensils and canned goods **(Wazir and Mokbel, 2019).**

Researchers have discovered that mustard contains higher levels of BPF compared to other bisphenols, indicating that certain foods may prefer one type of BPA alternative over others. Additionally, canned foods typically contain higher levels of bisphenols than foods stored in glass, paper, or plastic packaging **(Varghese and Hall, 2023).**

It is used to produce polycarbonate plastics, which are widely used in various everyday items such as optical devices, media storage, electrical appliances, housewares, medical equipment, dental supplies, and reusable or baby bottles. Additionally, it serves as a crucial ingredient in epoxy resins, primarily utilized for lining the interiors of food and beverage cans. Furthermore, it acts as an antioxidant or polymerization inhibitor in certain plasticizers, polyvinyl chloride, paper products, and thermal receipt papers.

BPA is also detected in dust, air, drinking water, surface water, and wastewater **(Srnovršnik et al., 2023),** It's been discovered in 86% of household dust samples, with concentrations ranging from 0.2 to 17.6 μg/g **(Ribeiro et al., 2017).** The primary route of human exposure to BPA is through consuming food and beverages stored in plastic packaging and bottles. It can be detected in various bodily fluids, including urine, serum, saliva, follicular fluid, breast milk, colostrum, placenta, umbilical cord, and amniotic fluid**(Srnovršnik et al., 2023).** Because of its resilience, flexibility, and durability, BPA has additionally been utilized in the production of weapons, safety gear such as helmets, and medical devices**(Santiago et al., 2021).**

IV. Exposure to BPA

The primary mode of exposure to BPA is through dietary intake, as exposure to temperatures exceeding 70 ◦C and the reuse of containers can lead to BPA seepage into food and beverages. Nevertheless, there's also a significant risk of exposure through inhalation and skin contact, particularly from thermal paper**(Santiago et al., 2021).**

While diet is recognized as the primary source of BPA exposure in humans, ingestion isn't the sole route of intake. BPA exposure can also happen through food and water consumption, dermal absorption from BPA-containing cash register receipts, and inhalation of air and dust, as shown in **(Figure 05)(Rubin et al., 2019).**

Moreover, BPA is believed to be transmitted from mother to offspring through intrauterine transfer during prenatal embryonic development and through breastfeeding in the early neonatal period**(Amjad et al., 2020).**

The estimated exposure levels range from 0.01 to 13 µg/kg/day in children and approximately 4.2 µg/kg/day in adults. However, even in fasting adults, BPA levels did not decrease over time, with concentrations exceeding 12 ng/mL still detected in urine after fasting for 6 or 9.5 hours. This indicates that BPA exposure from sources other than food and/or bioaccumulation in human tissue are significant contributors to overall BPA exposure**(Xu et al., 2016).**

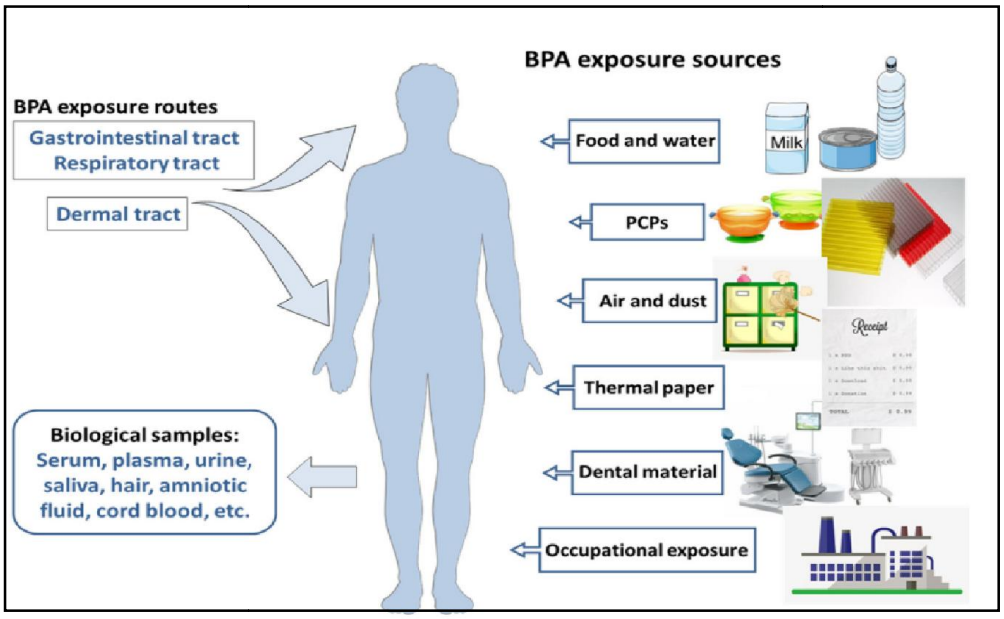

Figure 05: The routes and sources of BPA exposure inhumans, and the biological samplesof BPA detection**(Ma et al., 2019)**.

V. Metabolism of BPA

Extensive studies have been conducted on the metabolism of BPA using both in vivo and in vitro systems. BPA is present in human serum, urine, amniotic fluid, and breast milk in populations of industrialized countries across the globe.

V.1. Absorption and distribution

BPA can be absorbed through the skin and be a relevant internal exposure **(Mielke et al., 2011)**.Once orally ingested, BPA must pass through the digestive tract and liver before reaching the uterus or testicles.

V.2. Metabolism

BPA is primarily coupled to BPA mono-glucuronide by uridine 5′diphospho-glucuronosyltransferase (UGT) in the liver, forming BPA-GA, due to the presence of hydroxyl groups **(Mączka et al., 2022) (Nishikawa et al., 2010).**

Although the conjugated BPA does not bind to the estrogen receptor (ER), it can still disrupt cellular responsive action throughout membrane ERα contacts, which is responsible for quick signaling feedback.

On the other hand, in trace concentrations, unconjugated BPA can convert into other compounds such as BPA sulfate or BPA-S.BPA is not strongly conjugated with sulfuric acid by cytosolic sulfotransferase and thus does not require prior activation by cytochrome P450 (CYP) monooxygenases. However, CYP can metabolize BPA to bisphenol-o-quinone mediated by 5-hydroxy BPA, and the bisphenol-semiquinone intermediate. It is worth noting that both metabolites can bind to estrogen receptors.

V.3.Elimination

Conjugated BPA is eliminated primarily via urine, while unconjugated forms can undergo further metabolism before elimination. - BPA may negatively impact the hepatic metabolismof other xenobiotics, including drugs, by inhibiting the activity of certain CYP isoforms **(Manzoor et al., 2022)**.BPA stimulates the release of various hormones from the anterior pituitary gland, affecting peripheral tissues and potentially disrupting hormonal balance **(Santoro et al., 2019)**.

VI. Effects of BPA

VI.1.Human

Several studies have shown the importance of exposure to BPA during critical life stages like embryonic development and early life, which are considered the most vulnerable periods. This exposure is associated with effects on the female reproductive

system and carcinogenic potential. Male susceptibility to xenoestrogens like BPA is also a topic of debate in the scientific community, especially regarding sexual dysfunction and infertility**.**

BPA can pass through the placental barrier, reaching both maternal and fetal serum and placenta. It accumulates in different tissues, disturbing their normal functions. BPA triggers adipogenesis, encourages lipid buildup in adipose tissue and liver, and disturbs cytokine levels, leading to conditions like obesity, hypertension, and cardiovascular disease. Research suggests connections between BPA exposure and diabetes, anxiety, depression, and compromised immune function. Additionally, it may contribute to infertility, endometriosis, and premature puberty.

In addition, Data from cell line studies indicate that BPA disrupts the synthesis, secretion, and signaling of thyroid hormones. With its antiandrogenic activity, BPA behaves as both an estrogen receptor agonist and an androgen receptor antagonist. It exhibits teratogenic, mutagenic, and carcinogenic effects and can disrupt chromosome separation during meiosis, potentially leading to cancer susceptibility. Extensive research has linked BPA exposure to various cancers, particularly breast and ovarian cancers, primarily due to its hormone-like effects. However, emerging evidence suggests that BPA-induced reactive oxygen species (ROS) play a significant role in its toxicity and carcinogenicity **(Mączka et al., 2022).**

VI.2.Impact on the environment

Bisphenol is frequently found in wastewater, particularly from industrial facilities, due to its various applications. It's also detected in river sediments, notably in heavily urbanized areas. Both fresh and saltwater bodies contain BPA, which is absorbed by aquatic organisms. While BPA typically poses toxicity risks to aquatic life, certain species like the Mediterranean mussel Mytilusgalloprovincialis and the Greeneared Mussel Pernaviridis, demonstrate some resilience to its effects **(Mączka et al., 2022)**.

VII.Toxicity of BPA

VII.1.BPA and genotoxicity

The European Commission's health report stated that BPA doesn't cause genetic mutations. However, exposure to BPA at 100 μM or higher can harm cells, affecting their division and causing abnormal structures in HeLa cells, BPA exhibited genotoxic activity

in lab settings, but animal studies didn't find significant cancer-causing effects. Thus, BPA isn't considered genotoxic in living organisms.

DNA adducts weren't seen as a human health concern because there were no instances of gene mutation or chromosome abnormalities in cultured mammalian cells. Studies on both somatic and germ cells in living organisms also didn't confirm BPA's role in causing chromosome aberrations.However, the positive results from gene mutation tests conducted in bacteria and mammalian cells in the lab weren't either confirmed or ruled out by appropriate in vivo tests for the same genotoxic effects. As a result, a definitive conclusion regarding BPA's genotoxic potential couldn't be reached.Although there are inconsistencies, exposure to BPA has been found to cause DNA damage, both through its estrogenic properties and independently. These damaging effects on DNA are associated with the production of reactive oxygen species (ROS) **(Ribeiro et al., 2017).**

VII.2.Reproductive toxicity of BPA

Epidemiological research has demonstrated that BPA primarily affects the reproductive system, disrupting sex hormone function and impacting reproductive development and function. Studies indicate that BPA can elevate estradiol (E2), progesterone, serum luteinizing hormone (LH), and testosterone (T) levels while decreasing serum cortisol concentrations. Although some findings suggest a significant association between BPA exposure and higher total testosterone (TT) levels, other studies have shown BPA to reduce T levels and increase E2 concentrations.

Fluctuations in sex hormones impact endometrial wall thickness. Researchers noted a link between endometrial thickness and BPA levels, with age playing a role in this association. In women under 37, higher urinary BPA levels correlated positively with endometrial thickness, while in those 37 or older, endometrial thickness decreased with higher BPA concentrations in urine.

Moreover,Polycystic ovary syndrome(PCOS) patients exhibit higher serum BPA levels compared to healthy women and adolescents. This suggests a potential involvement of BPA in the development of PCOS**(Ma et al., 2019).**

VII.3.Brain toxicity

The main concerns involve understanding the immediate and long-term impacts of BPA (and similar substances) on the brain. As the control center of the body, the brain integrates information and swiftly responds to changes in the environment and physiology. While a complex network of neurons forms during fetal development, full brain maturity

occurs postnatally. Therefore, safeguarding neuronal cells and synaptic flexibility is crucial for brain health, whereas neuronal damage, inflammation, and synapse loss contribute to neurological disorders and cognitive decline.

Exposure to BPA in the environment can disrupt brain development and function by evading the body's natural defenses, potentially necessitating external interventions. Specifically, the hypothalamus reacts to internal and external signals such as energy levels, stress, temperature changes, and hormonal fluctuations**(Santoro et al., 2019).**

Chapter III

Triclosan

I. Definition of triclosan and its properties

Triclosan (TCS) is a highly effective antibacterial and antimicrobial agent widely employed in various applications. It is commonly found in personal care items like toothpaste, antimicrobial soap, skin creams, and everyday consumer goods such as clothing, plastic kitchenware, sports shoes, and socks.

Additionally, it is utilized in medical supplies including dental care products, medical preservatives, and bactericides, as well as in household cleaning products like detergents and disinfectants. Commercially, it goes by several names including Irgasan, DP300, FAT 80'023, CH 3565, and GP 41-353**(Malashetty et al., 2021).**

Triclosan, initially developed by the Ciba-Geigy Company in Basel, Switzerland, during the early 1960s **(Jones et al., 2000)**, is characterized as a halogenated aromatic hydrocarbon. Its chemical composition comprises phenolic, diphenyl ether, and polychlorinated biphenyl (PCB) substructures, as illustrated in (**Figure 06**).

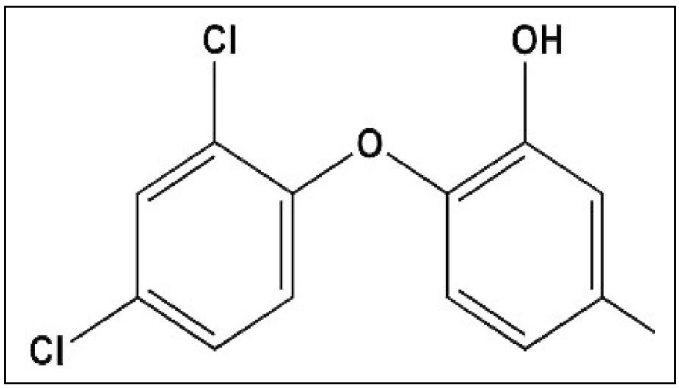

Figure 06. Molecular structure of triclosan **(Olaniyan et al., 2016)**.

Table 1. Physical and chemical properties of triclosan**(Lee et al., 2019;Marques et al., 2022).**

Property	Value and conditions
INCI name	Triclosan
IUPAC name	5-Chloro-2-(2,4-dichlorophenoxy)phenol
CAS No	380-34-5
Molecular formula	C12H7Cl3O2
Molar mass	289.54 g/mol
Physical state and odor	White to off-white crystalline powder with a slight, faintly aromatic odor
Nature	Hydrophobic/lipophilic
Melting point	55-57°C
Boiling point	280-290°C (decompose)
Density	1.49 g/cm3
Water solubility	0.012 g/L at 20°C
Log Pow	4.76
Synonyms	2,4,4'-Trichloro-2'-hydroxy-diphenyl ether
GHS Identification (General Harmony System)	 IrritantEnvironmental
Triclosan degradation products	Methyl-TCS, dioxin, chlorophenol, chloroform

II.Application of triclosan

TCS typically presents as a white powder with a subtle phenolic odor, owing to its composition as a chlorinated aromatic compound. Since its inception, this compound has been extensively utilized across a wide array of consumer products, as outlined in (**Figure 07**).

Its adoption dates to the 1980s in Europe and the mid-1990s in the United States, following approval by the Food and Drug Administration (FDA) particularly in dental products.

More precisely, TCS finds application in numerous personal care items such as toothpaste, antibacterial soaps (both in solid and liquid forms), dishwashing liquids, deodorant soaps (both solid and liquid variants), as well as cosmetics, antiseptics, and antiperspirants/deodorants. Additionally, it is incorporated into various other consumer goods including kitchen utensils, toys, bedding, clothing, fabrics, and trash bags**(Dhillon et al., 2015)**.

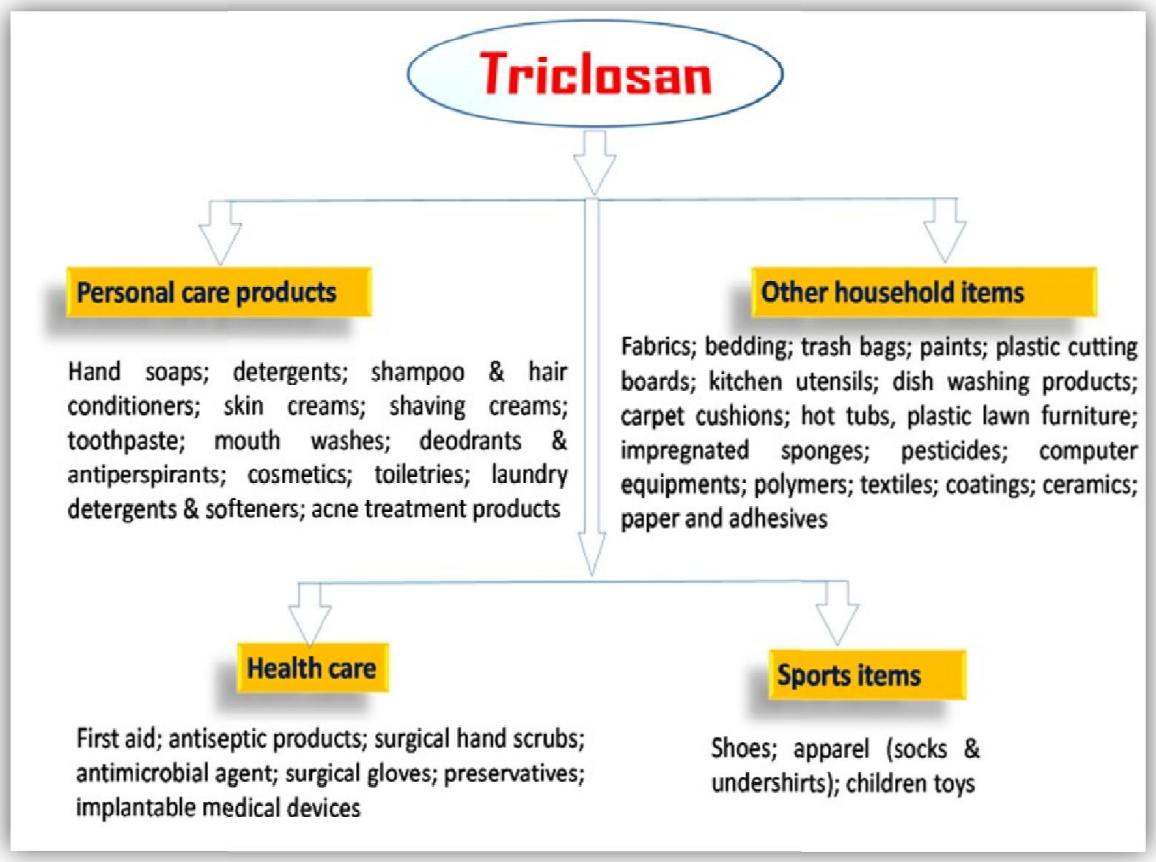

Figure 07. Various applications of triclosan**(Dhillon et al., 2015)**.

The concentration of TCS advised by different governmental bodies for use in various consumer products is detailed in **Table 2**. In 1989, the European Community Cosmetic Directive sanctioned TCS utilization as a preservative in cosmetics and toiletries, up to 0.3%. According to FDA guidelines, toothpaste can contain TCS up to 0.3%. Likewise, as stated by the National Library of Medicine's Household Products Database, TCS concentrations in liquid hand soaps were documented to vary between 0.1% and 0.3%.

Table 2. Recommended levels of TCS in various consumer products **(Dhillon et al., 2015)**.

Type of TCS-Based Product	TCS Concentration (%)
Oral care products	
Toothpaste	0.3
Mouth wash solutions	0.03
Dermally applied products (rinse off)	
Skin cleansers	0.3
Liquid hand soap	0.1–0.45
Dishwashing detergent	0.1
Dermally applied products (leave on)	
Body lotion	0.3
Facial Moisturizer	0.3
Deodorant/antiperspirants	0.3

III.Metabolism of triclosan

III.1. Absorption

TCS can be taken into the body through various pathways, including absorption through the oral mucosa, skin contact, or ingestion via the gastrointestinal tract. Given that many TCS-containing products are designed for application to the skin, the primary method of absorption is through the skin, where it is quickly taken up owing to its affinity for fats.

Additionally, triclosan can be absorbed orally, leading to the detection of its metabolites in plasma, as well as through the gastrointestinal tract. Concentrations in the millimolar range have been observed in humans. Therefore, considering the prevalent use of TCS in consumer products, all pathways of exposure are significant.

In a 1992 study, it was found that when 1.6 mg of triclosan was directly applied to the skin of rats, it was swiftly absorbed, with peak concentration occurring between 12- and 18 hours post-exposure. Subsequent research by Moss, Howes, and Williams indicated that triclosan permeates rat skin more rapidly than human skin. After 24 hours, only about 12% of the dose remained on human skin compared to about 26% on rat skin**(Marques et al., 2022).**

III.2. Distribution

Samples from humans exposed to TCS through consumer products were collected and analyzed, revealing that the liver had the highest concentration of TCS, followed by adipose tissue, with the brain showing the lowest concentration. Upon applying TCS to the

skin of mice, it was detected in all 15 tissues tested 12 hours later, with the gall bladder, bladder, and skin application site having the highest amounts, and the testes, thymus, and brain having the lowest levels. In male rats, TCS did not accumulate in the prostate, but high levels were found in the epididymis. In mice undergoing percutaneous absorption, the gall bladder and liver exhibited the highest accumulations, with concentrations of 402 and 10.5 μg/g tissue, respectively**(Lm and Ja, 2017)**.

III.3. Metabolism

When it comes to metabolism, TCS can be processed through three pathways: cytochrome P450, UDP-glucoronyltransferase (UGT), and sulphotransferase (SULT) shown in (**Figure08**). The latter two are involved in the phase II metabolism of xenobiotics.

The primary metabolite resulting from TCS hydroxylation is triclosanmonohydroxylate. This can then be broken down into 2,4-dichlorophenol and 4-chlorocatechol. Additionally, a fourth metabolite can be formed from the hydroxylation of 2,4-dichlorophenol, producing 3,5-dichloro catechol. Although the two pathways of phase II metabolism are unaffected by the route of administration, the ratio between the transformation of TCS by these pathways is influenced by the species. Glucuronidation and sulphation of TCS add glucuronic acid and sulfate, respectively, to thehydroxyl group, ultimately destroying the proton translocating nature and adding a charged/polar group to TCS. This enhances its hydrophilic properties and reduces TCS's ability to accumulate in adipose tissue.

The liver is the primary site of TCS metabolism, with minimal levels also occurring in the skin. TCS is primarily metabolized through the SULT pathway, but phase II metabolites are present 24 hours after administration. Despite undergoing metabolization, the unmetabolized form of TCS remains the dominant form. In the liver, metabolite production depends on the TCS dosage. For exposures of 1–5 μM of TCS for 30 minutes, TCS-sulphate and TCS-glucuronyl are equally produced. For doses greater than 20 μM, glucuronidation is dominant, while for doses below 1 μM leads to sulphation. A 2014 study involving 46 volunteers (26 men and 20 women aged 4 to 80 years) found both TCS-sulphate and TCS-glucuronyl in urine samples, with TCS-glucuronyl being the primary metabolite detected**(Marques et al., 2022).**

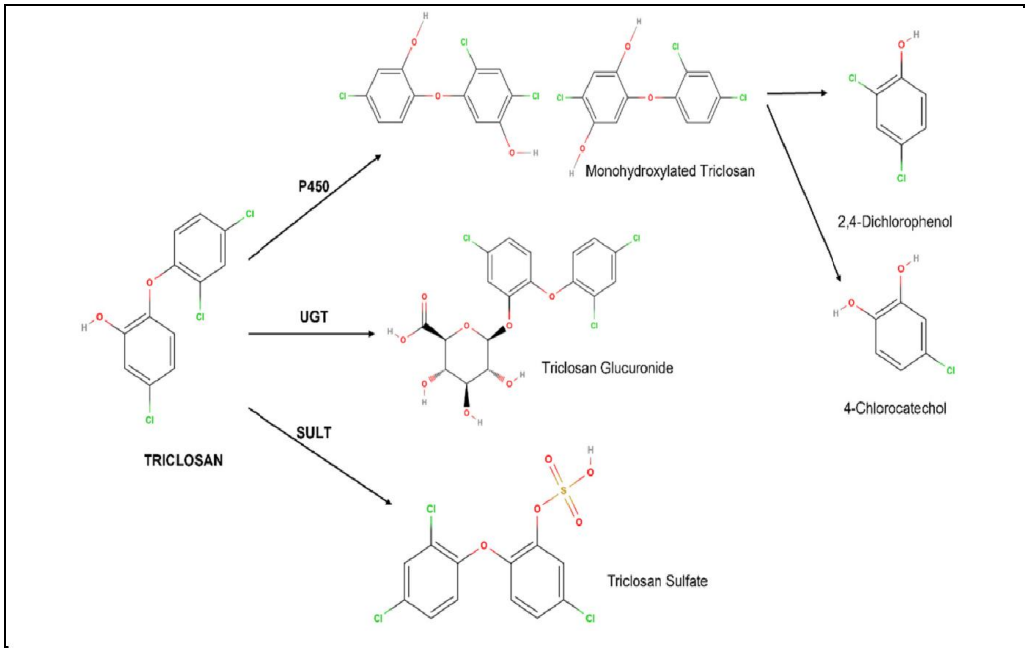

Figure 08. Metabolism of triclosan and its metabolites **(Marques et al., 2022).**

III.4.Elimination

Triclosan is primarily eliminated from the human body through urine, with fecal elimination being the secondary route. It is excreted mainly in its conjugated form. After oral exposure, urinary excretion increases within 24 hours, with 24 to 83% of the consumed TCS excreted during the first 4 days post-exposure. After 8 days, excretion levels return to baseline, with a half-life of 21 hours following oral exposure.

In rats, topical exposure resulted in 12% of the dose being excreted through feces and 1% through urine. Siddiqui (1979) noted a rapid transfer of TCS from blood to tissues and found that the compound did not accumulate in long-term storage compartments such as fat in the body**(Weatherly and Gosse, 2017)**.

IV.Human and animal impact

In vivo human toxicity of TCS has not been precisely demonstrated, butdetectable levels of TCS have been found in various bodily fluids and tissues of exposed individuals, including blood, breast milk, urine, adipose tissue, brain, liver, and nails. This suggests that TCS may have an impact on human physiology. The higher concentrations of TCS in tissues compared to environmental levels suggest potential bioaccumulation and widespread distribution in human tissues. Allergic reactions in humans have been reported, such as dermatitis from prolonged use of TCS-containing hand washes or toothpaste and

blistering on the mouth and lips after TCS exposure. Epidemiological studies have linked increased TCS levels in urine to immune dysfunction, allergic reactions, and asthma in children. Recent laboratory experiments have demonstrated that TCS can interact with human serum albumin, leading to a change in the protein's conformation. When harmful substances bind to serum albumin, it can hinder the transportation of natural substances and cause a change in the protein's molecular structure which may affect its activity or even change its physiological function **(Olaniyan et al., 2016)**.

The toxic effects of TCS have been investigated across various animal models. For example, in mice, it disrupts thyroid hormone metabolism, leading to hypothermia and CNS depression. Exposure to 0.03 mg·L−1 of TCS prompts premature metamorphosis in tadpoles by inducing the expression of metamorphic genes. Similarly, research by Kumar et al. links TCS exposure to reduced sperm production in male rats, suggesting TCS may interfere with thyroid hormone metabolism by binding to specific receptors due to its structural similarity.

In chronic oncogenicity studies involving mice, rats, and hamsters, liver tumors were exclusively observed in both male and female mice. While the human relevance framework suggests these tumors may not directly translate to humans, Yueh et al. found that prolonged TCS exposure in mice enhances hepatocellular carcinoma **(Dhillon et al., 2015)**.

TCS has been detected in both environmental samples and animals. In Germany, fish samples from rivers showed TCS concentrations in fish muscle tissue ranging from 4.6 to 18.6 ng/g (lipid weight), indicating its accumulation in fish tissue **(Rüdel et al., 2013)**.Similarly, researchers in the U.S. found TCS in salmon tissue at a concentration of 25 ng/g (wet weight).

Additionally, earthworms exposed to biosolid application for four years exhibited TCS concentrations ranging from 15.7–51.4 ng/g (dry weight)**(Lm and Ja, 2017)**.

V.Effects of triclosan on male reproductive health

Exposure to TCS resulted in negative impacts on the sperm and reproductive organs of mammals. Research indicated that TCS tended to accumulate in the epididymis of male Sprague Dawley (SD) rats. Rats treated with high doses (200 mg/kg) of TCS exhibited a notable decrease in daily sperm production (DSP), alterations in sperm morphology, and an increase in the quantity of malformed sperm.

Furthermore, TCS could directly or indirectly affect hormone receptor expressions and hinder testicular steroidogenesis, consequently disrupting male reproduction and fertility **(Chen et al., 2023).**

VI.Effects of triclosan on female reproductive health and offspring

Exposure to TCS in females not only affects levels of reproductive hormones but also disrupts ovarian function and can impact subsequent generations. TCS exposure disrupts energy metabolism by altering glucose flow, leading to disturbances in steroid hormone production and hormone balance, thus affecting female reproductive development.

In adult female mice, TCS exposure decreased thyroid hormone levels, resulting in elevated prolactin levels, and subsequently suppressing hypothalamic kisspeptin expression. This sequence of events ultimately leads to disruptions in reproductive hormone balance and functional defects in the reproductive system. The adverse reproductive effects reported in animal studies were similar.In mice, high TCS concentrations were linked to spontaneous abortions **(Wang et al. 2015)**.

Administering TCS during specific gestational periods resulted in decreased implantation rates in mice. Pregnant rats exposed to TCS showed elevated rates of spontaneous abortion, and the placenta was observed to accumulate the compound post-treatment**(Lm and Ja, 2017)**.

VII.Effect of triclosan on the thyroid system

Numerous chemicals that disrupt the endocrine system can have negative effects on the thyroid hormone system. These chemicals can lower thyroid hormone levels in various ways, such as by inhibiting iodide uptake and thyroid hormone synthesis in the thyroid gland. However, there are other ways in which the thyroid hormone system can be disrupted, and more chemicals have been found to interfere with enzymes, thyroid hormone receptors, and serum distribution proteins and transporters that are important in mediating thyroid hormone action. The significance of a properly functioning thyroid for normal metabolic functions and brain development is well-known, but the full extent of the impact of chemicals present in household and cosmetic products on the endocrine system is not entirely understood. Studies have explored the possible effects of triclosan on thyroid hormone levels in humans, focusing mainly on the pituitary hormone TSH and thyroid hormones T4 and T3. T4 is produced and released in greater quantities than the more

active T3, and most of it is converted to either T3 or the inactive reverse T3 in peripheral tissue cells. T3 can then bind to and activate nuclear receptors. As such, T4 is considered a prohormone. Serum hormone measurement options include total T4 and T3, which account for both bound and free hormones, or estimation of free hormone concentrations (fT4 and fT3), representing a small portion of circulating hormones. The low concentrations and protein binding contribute to measurement variability, along with potential disturbances from physiological, pathophysiological, and pharmacological factors **(Homburg et al., 2022)**.

Oral administration of TCS resulted in decreased hormone levels, such as progesterone, estradiol, and testosterone, in rat serum show (**Figure 09**). Female rats showed lowered thyroxine (T4) hormone levels after oral TCS intake, with similar decreases observed in pups and male juvenile rats exposed to TCS **(Lm and Ja, 2017)**.

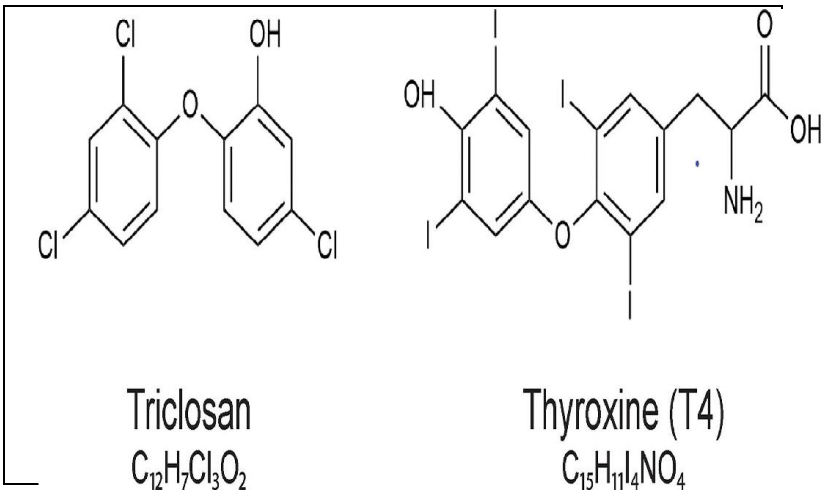

Figure 09. The chemical structures of triclosan and the thyroid hormonethyroxine (T4) **(Homburg et al., 2022)**.

VIII.Environmental impact

Most antibacterial personal care products are rinsed off and disposed of as household waste. While wastewater treatment plants remove some TCS, a small amount still enters water bodies. Consequently, TCS and its harmful byproducts, including methyl triclosan, dioxins, chlorophenols, and chloroform, are frequently found in aquatic environments, sediments, biosolids, soils, and organisms. In the USA, TCS concentrations in natural streams and rivers typically range from undetectable to 2300 ng/L, usually below 100 ng/L. The presence of antimicrobials in the environment, even at low levels, poses a hazard due to their high lipophilicity, potentially leading to bioaccumulation. TCS

accumulation in aquatic organisms like algae, crustaceans, shellfish, fish, and marine mammals is well-documented. Additionally, aquatic organisms appear to be particularly sensitive to adverse reproductive and developmental effects caused by TCS **(Ruszkiewicz et al., 2017)**.

IX.Triclosan degradation products

Triclosan undergoes degradation through both biological and non-biological processes. Triclosan-methyl (mTCS) is a product of TCS degradation, primarily through biodegradation. Under aerobic conditions, TCS-methyl is formed in the environment via O-methylation, where a methyl group attaches to the hydroxyl group on the TCS molecule. This transformation is facilitated by certain bacteria like Rhodococcus, Acinetobacter, and Mycobacterium, like other chlorophenolic compounds.

Triclosan-methyl has been detected in treated wastewater treatment plant (WWTP) effluent, river water, and soil. Additionally, TCS degradation to mTCS occurs within WWTPs. However, WWTPs partially limit total TCS removal by converting the parent compound to mTCS, which is more resistant to photolysis.

The biological transformation of TCS also yields various by-products such as 2,4-dichlorophenol, 4-chlorophenol, 2-chlorohydroquinone, catechol, and hydroquinone, depending on the microbial composition shown in (**Figure 10**) Optimal biotransformation conditions occur at a pH of 7 and a temperature of 30°C.

Moreover, abiotic photodegradation of the dissociated form of TCS results in multiple breakdown products, including dioxins. Rapid abiotic photodegradation of TCS at high pH leads to the formation of 2,8-dichlorodibenzo-p-dioxin (DCDD). The conversion of TCS to DCDD varies depending on pH and light wavelength but ranges from 1-12%, indicating that while DCDD is a significant by-product of TCS degradation, it is not the predominant one.

Abiotic transformation of TCS also results in the generation of chlorophenols, which represent a significant category of byproducts formed when TCS undergoes chemical treatments. When TCS is exposed to chlorine, it can degrade into 4,5 dichloro-2-(2,4-dichlorophenoxy)phenol (4-ClTCS), 5,6 dichloro-2-(2,4-dichlorophenoxy)phenol (6-Cl-TCS), and 4,5,6 trichloro2–2(2,4dichlorophenoxy)phenol (4,6-Cl-TCS)**(Weatherly and Gosse, 2017)**.

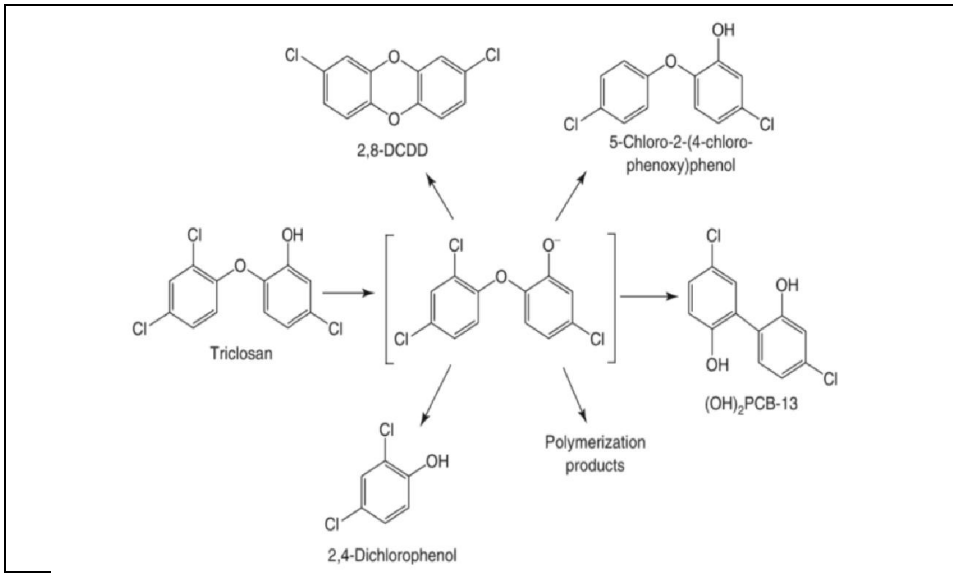

Figure10. Photochemical degradation products of triclosan **(Khetan, 2014)**.

Experimental part

Chapter IV
Material and Methods

This study aims to examine the sub-chronic toxicity effects of daily supplementation of a combination of two endocrine disruptors, bisphenol A (BPA) and triclosan (TCS) in female Wistar rats. The research was conducted exclusively within the animal housing facility and the biochemistry laboratory of the University of 8 Mai 1945, as well as the anatomopathological department of Ibn Zohr Hospital in Guelma.

1. Material

1.1. Animals

The study was conducted on female albino Wistar rats obtained from the animal house at the University of 8 May 1945, Guelma. During the acclimatization period, the rats weighed between 130 and 150 grams, and at the time of the experiment, their average weight ranged from 145 to 200 grams. The animal testing was performed according to the ethical standards approved by the European Community Council Directive of 24 November 1986 (86-609/EEC) and the Decree of 20 October 1987 (87-848/EEC).

1.2. Conditions for Accommodation

The rats were housed in metal cages lined with bedding composed of wood chips. The cages were cleaned, and the bedding replaced daily. The environment in the animal facility was maintained at a temperature of 25°C, with natural humidity, and a photoperiod resembling spring. The rats were fed a diet of corn and barley pellets, and drinking water was provided in bottles adapted to the cages.

1.3. Treatment of Animals

After the adaptation period, the female rats were divided into six groups:

- ✓ **Group 1** (vehicle control n=5): 5% ethanol and 95% corn oil (0.3 cc/day) were administered subcutaneously for 25 consecutive days.
- ✓ **Group 2** (low-dose BPA, n=6):BPA at 50 µg/kg/day was administered intraperitoneally for 25 consecutive days.
- ✓ **Group 3** (low-dose TCS n=8): TCS at 01 mg/kg/day was administered subcutaneously for 25 consecutive days.
- ✓ **Group 4** (low-dose BPA/TCS, n=10):BPA at 50 µg/kg/day was co-administered intraperitoneallywith TCS at 1 mg/kg/day subcutaneously for 25 consecutive days. BPA and TCS, in the form of dry powder, were dissolved in 5% ethanol and suspended in corn

oil based on the procedure found in the literature **(Li et al., 2016)(Montagnini et al., 2018).**

- ✓ **Group5**(high-dose BPA n=6): BPA at 100 mg/kg/day was administered intraperitoneally for 25 consecutive days.
- ✓ **Group 6** (high-dose TCS n=10):TCS at 01 mg/kg/day was administered subcutaneously for 25 consecutive days.
- ✓ **Group 7** (high-dose BPA/TCS, n=6): BPA at 100 mg/kg/day was co-administered intraperitoneally **(Li et al., 2016)**with TCS at 10 mg/kg/day subcutaneously for the same duration(**Montagnini et al., 2018)**.
- ✓ **Group 8** (WWTP n=5): Rats were provided with treated water collected from WWTP *ad libitum* for 25 days. This group aimed to compare toxic effects observed in previous groups with the effects of exposure to treated water. Previous research reported that BPA was not fully eliminated (approximately 23%, increasing to 31% at the end of the processing chain) from water in Guelma WWTP.

1.4. Sacrifice and Collecting of Blood and Organs

After sacrifice, blood is collected from the heart using the cardiac puncture technique for biochemical analysis**(Christine et al., 2007).** Subsequently, organs including the liver, kidneys, and ovaries are extracted, preserved in a freezer at -20°C, and homogenized on the designated day. Tissue samples (liver, kidney, and ovaries) from each rat in various experimental groups are ground and homogenized in a saline phosphate buffer (0.1 M, pH 7.4).

These organ homogenates are utilized to assess oxidative stress markers, including malondialdehyde (MDA), reduced glutathione, advanced oxidative protein products, glutathione peroxidase activity, and tissue protein levels.

2- Methods

2.1 Preparation of the Cytosolic Fraction

After the reperfusion period, a segment of the liver, kidney, and ovaries was excised for histopathological examination. The remaining tissue from these organs was weighed, fragmented into small pieces, and submerged in three volumes of phosphate buffer (0.1 M, pH 7.4 at 4°C) containing KCl (1.17%). Subsequently, homogenization was performed using an ULTRA-TURRAX homogenizer. To this homogenate, 0.5 mL of trichloroacetic acid (5% TCA) was added in equal volume and then centrifuged at 4°C for

10 minutes at 4000 rpm. The resulting supernatant was utilized for the reduced glutathione assay.

A portion of this supernatant was allocated for the MDA assay, while the remaining portion underwent centrifugation at 4°C for 45 minutes at 10,000 rpm. The resultant supernatant was then utilized for the assessment of antioxidant enzyme activity.

2.2 Relative Organ Body Weight variation

The relative organ body weight, which assesses the proportion of an organ's weight relative to the overall body weight, is calculated by dividing each animal's organ weight by its body weight. This calculation provides insights into organ development and the potential effects of sub-chronic exposure to BPA and TCS on organ size. The formula for calculating the relative organ body weight can be expressed as follows:

Relative Organ Body Weight = Absolute Organ Weight / Body Weight

2.3 Blood Glucose Level

Two hours before sacrifice, blood samples were collected from the tail of the rat and used to determine serum glucose activity using a glucometer.

3. Oxidative stress biomarkers

3.1. Evaluation of Lipid Peroxidation (MDA)

Lipid peroxidation is a well-established mechanism of cellular injury in animals; it is used as an indicator of oxidative stress in cells and tissues. In this test the reaction of lipid peroxides with TBA makes a complex that was determined spectrophotometrically, and lipid peroxidation was assessed in terms of thiobarbituric acid reactive substances TBARS produced.

The malondialdehyde (MDA) level, regarded as a final product of lipid peroxidation, was quantified following the colorimetric method outlined by **(Ohkawa et al., 1979).**

In this method, 0.25 ml of the supernatant containing MDA reacted with 500 µL of thiobarbituric acid (TBA) solution (0.67%) in the presence of 0.25 ml of trichloroacetic acid (TCA 20%). The reaction mixture underwent incubation at 100°C for 15 minutes, followed by cooling. The formation of a pink compound occurred, which was then extracted by adding 2 ml of n-butanol. Subsequently, the organic phase containing the complex formed between MDA and TBA was separated through centrifugation at 3000 rpm for 15 minutes. The absorbance of the resulting solution was measured at 532 nm using a Jenway 6305 UV/visible spectrophotometer. TBARS concentration of the sample

was calculated using 1.56 x 10^{5} M^{-1} x cm^{-1} as the extinction coefficient of MDA (since 99% of TBARS exists as MDA).

3.2. Evaluation of Protein Oxidation

The level of protein oxidation in the three tissues was assessed by measuring advanced protein oxidation products (AOPP) quantified by a spectrophotometric method described by **(Witko-Sarsat et al., 1996).**

To assess the AOPP content, 0.2 mL of the supernatant was diluted in 0.4 mL phosphate buffer (0.1 M, pH 7.4) and combined with 50 µL of potassium iodide (1.16 M). After two minutes, 0.1mL of glacial acetic acid was introduced, and the mixture underwent centrifugation at 3500 × g for 15 minutes at 4°C. The absorbance of the sample below the lipid phase was then measured at 340 nm.

The three-tissue AOPP content was calculated using the extinction coefficient and expressed as nmol/mg protein. It is determined using the following formula:

$$\mathbf{AOPPRate} = \mathbf{DO} \times \frac{\mathbf{DF}}{\boldsymbol{\varepsilon} \times \mathbf{L} \times \mathbf{CP}}$$

DO: Optical density of the sample at 340 nm.

Δt : time interval in minutes.

DF: Dilution factor (Vt/Vs) or Vt: Total volume of the reaction medium, Vs: Supernatant volume.

□: Molar extinction coefficient of AOPP (261mM^{-1} cm^{-1}).

L: Length of tank used (L = 1 cm).

CP: Protein concentration (mg/g of tissue).

3.3. Reduced Glutathione Assay (GSH)

Ellman's method was utilized to assess hepatic cytosolic glutathione levels **(Ellman, 1959)**This method relies on the oxidation of (GSH) by 5,5-dithio-2-nitrobenzoic acid (DTNB), resulting in the release of 2-nitro-5-mercaptobenzoic acid. This compound exhibits an absorbance of 412 nm at alkaline pH.

850µL of phosphate buffer (pH 7.4, 0.1 M) is added to 100 µL of the supernatant. Following this, 50 µL of a DTNB solution (0.01 M prepared in phosphate buffer) is added. After shaking and incubating for 5 minutes at room temperature, the optical density of the

resulting supernatant is measured using a spectrophotometer at 412 nm against a blank prepared under the same conditions with distilled water.

The GSH level is determined using a standard curve prepared with GSH at increasing concentrations ranging from 0.2 to 1 mM. The results are expressed as micromoles per gram of tissue.

3.4. Glutathione Peroxidase Enzymatic Activity Assay (GSH-Px)

The enzymatic activity of glutathione peroxidase (GSH-Px) was measured by the method **(Flohé and Günzler, 1984)**. This method relies on the reduction of hydrogen peroxide (H_2O_2) in the presence of reduced glutathione (GSH). The GSH is transformed into its oxidized form (GSSG) under the influence of GSH-Px according to the following reaction:

$$GSH + H\square O\square \xrightarrow{GSH-Px} GSSH + H\square O\square$$

The enzymatic activity of glutathione peroxidase (GSH-Px) was measured using the method described by **Flohé and Günzler (1984)**. This method relies on the reduction of hydrogen peroxide (H_2O_2) in the presence of reduced glutathione (GSH), transforming GSH into its oxidized form (GSSG) under the influence of GSH-Px. To measure the enzymatic activity, 400 µL of reduced glutathione (GSH) at a concentration of 0.1 mM was added to 200 µL of the supernatant. Subsequently, 200 µL of a TBS buffer solution containing Tris (50 mM) and NaCl (150 mM) at pH 7.4 was added. The mixture was incubated for 5 minutes in a water bath at 25°C. After incubation, 200 µL of hydrogen peroxide (H_2O_2) at a concentration of 1.3 mM was added to initiate the reaction, and the mixture was allowed to react for 10 minutes. Following the reaction, 1 mL of trichloroacetic acid (TCA) at a concentration of 1% was added to stop the reaction. The mixture was then placed on ice for 30 minutes. After incubation on ice, the mixture was centrifuged for 10 minutes at 3000 rpm. Then, 2.2 mL of TBS buffer solution was added to 480 µL of the supernatant. Finally, 0.32 mL of DTNB (1.0 mM) was mixed in, and the optical densities were measured at 412 nm after 5 minutes.

$$GSH - Px = \frac{\text{DO ec} \times \text{DO et} \times \text{DF}}{\text{DO et} \times \text{CP}} 0.04$$

3.5. Tissue Protein Assay

The protein content of samples from three different organs was determined using the biuret method. This method, known for its sensitivity and rapidity, measures the quantity of peptide bonds through the absorption of a dye **(Gornall et al., 1949).**

For the assay, 1 mL of biuret reagent is added to 20 µL of each supernatant sample. The mixture is stirred and then incubated for 5 minutes at 37°C. The optical density of the reaction mixture is then measured at 540 nm against a blank prepared with the reagent. A standard solution containing 7 g/dL of BSA is used as a reference show **(Appendice 1)**

The protein concentration expressed in g/dL in the samples is calculated using the absorbance readings with the following formula

$$g/dl\text{totalprotein} = \frac{\text{Asample}}{\text{Astandard}} \times \text{C standard}$$

4. Histopathological Analysis

In this phase of the study, the samples were fixed in a solution containing 10% formaldehyde to preserve their structural integrity. Subsequently, they were impregnated and embedded in paraffin blocks to facilitate thin sectioning. After embedding, 5µm-thick sections were obtained and stained with hematoxylin-eosin (H&E) to highlight cellular structures.Visual examination of any lesions affecting the tissues of the three organs was conductedusing an optical microscope, specifically the Olympus C41 model. The histological sections were observed at two magnifications (10x and 40x), enabling a detailed analysis of any abnormalities present.

5. Statistical Analysis

All data were presented as mean ± standard error of the mean (S.E.M.). Statistical analysis was conducted using GraphPad Prism version 10.2.3 (403) software. Differences between groups were assessed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post-test. A significance level of $p < 0.05$ was considered statistically significant to interpret the differences in the results of the biochemical parameters.

Chapter VI

Discussion

Triclosan is widely used as an antimicrobial agent in personal care products, while Bisphenol A is a common industrial chemical found in plastics and resins. Both compounds have been shown to interfere with hormonal functions, raising concerns about their combined impact on health. These pollutants can interfere with processes regulated by endogenous hormones**(Alonso-Magdalena et al., 2006).**

It has been reported that the ability of BPA to bind oestrogen receptors may cause various disorders including hypertension, atherosclerosis, liver dysfunction, diabetes, and obesity **(Marmugi et al., 2014)**. On the other hand, Triclosan was described as a poor potential endocrine disruptor as it has been discovered to bind the androgen and estrogen receptors with agonistic and antagonistic effects**(Wang and Liang, 2021)** triclosan can interfere with estrogenic (ER) and androgenic (AR) receptors and it has adverse effects on the thyroid and cardiovascular systems(Marques et al., 2022b)**.**

In the present study, we aimed to assess the potentially toxic effects of subchronic co-exposure to those two endocrine perturbators with different doses aiming to elucidate their potential synergistic effects by exploring biochemical and histological changes in the liver, the kidney, and the ovary of female Wistar rats.

Our analysis began with evaluating the relative organ body weight, a crucial parameter for assessing potential toxicological effects, as changes in organ weight can indicate tissue-specific toxicity or systemic stress. Contrasting our findings of a decrease in liver weight at a low dose of BPA/TCS and an increase in ovary weight with a high dose of BPA/TCS, **Baralić et al., 2020** reported increased liver, kidney, and testicle weights under high-dose mixtures (50 mg/kg b.w. DEHP (bis 2-ethylhexyl phthalate), + 50 mg/kg b.wand DBP (dibutyl phthalate) + 25 mg/kg b.w BPA) after 28 days of oral treatment, highlighting complex dose- and mixture-dependent effects on organ weights.

Then, the variation of glycemia in the treated animals was assessed to confirm whether endocrine disruptors such as triclosan (TCS) and bisphenol A (BPA) can interfere with the hormonal regulation of glucose metabolism. In our study, the administration of these compounds at subacute levels led to significant variations in glycemia among the treated female Wistar rats with the low-dose combination of BPA and TCS (50 µg BPA +1 mg TCS per kg of body weight) and in those exposed to both low and high doses of BPA alone. This suggests that the combined exposure to TCS and BPA may disrupt normal endocrine function, particularly affecting hormones like insulin and glucagon which are

critical for maintaining glucose homeostasis. The observed glycemia variation underscores the potential for these chemicals to induce metabolic disturbances, further highlighting the subacute toxic effects of these endocrine disruptors.

Surprisingly, our findings indicate no significant change observed in the blood glucose levels of animals exposed to high-dose BPA/TCS compared to the control. In contrast, the study by **(Amraoui et al., 2018)**showed that rats treated orally with 10 mg/kg b.w. of bisphenol A for 3 weeks had elevated glucose levels and experienced hepatic disorders and hyperglycemia. Additionally, **Baralić et al. (2020**)reported increased glucose levels in rats exposed to a mix including BPA, DEHP (bis 2-ethylhexyl phthalate), and DBP (dibutyl phthalate), suggesting complex interactions in combined chemical exposures.

Concerning the implication of oxidative stress, results from various studies consistently highlight the significant impact of endocrine-disrupting chemicals on oxidative stress and lipid peroxidation in rats.

Our findings highlight significant membrane damage in the liver, kidney, and ovaries of rats subjected to the highest doses of bisphenol A (BPA), as evidenced by markedly increased levels of malondialdehyde (MDA), a key indicator of lipid peroxidation. In contrast, when high doses of BPA and Triclosan (TCS) were combined, a pronounced rise in MDA levels was observed in the ovaries, with no significant changes detected in the liver and kidney, suggesting tissue-specific oxidative stress responses. This is consistent with other studies that illustrate the varying organ susceptibilities to oxidative stress induced by similar compounds. For instance, **(Oluranti et al., 2023)**found that co-administration of dibutyl phthalate (DBP) and BPA significantly elevated renal MDA levels, while **Riad et al., 2018**reported increased testicular MDA content following a regimen of butylparaben (BP) and Triclosan. Additionally, **Amraoui et al. (2018)**confirmed significant MDA elevation in rats exposed to BPA alone. These studies collectively underscore that lipid peroxidation, as marked by increased MDA, serves as a common biochemical pathway through which various endocrine disruptors exert their damaging effects, albeit with organ-specific variances in susceptibility and response.

Advanced oxidation protein products (AOPPs) are crucial indicators of oxidative stress and can provide insights into the body's oxidative state. They are generated during

oxidative stress conditions, where there is an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defenses.

In our work, we observed significant changes in AOPP levels in rodents exposed to subacute doses of TCS and BPA. Emphasizing a dose-dependent oxidative impact of the two xenobiotics on specific organs: high doses significantly elevated AOPP levels in both the liver and ovaries, while low doses affected only the ovaries, indicating organ-specific sensitivity to oxidative stress at different exposure levels. In contrast, **(Baralić et al., 2020)**reported no significant changes in AOPP levels following a 28-day combined exposure to DEHP, DBP, and BPA at considerable doses, suggesting that mixed chemical exposures might not produce additive oxidative effects. This could be due to possible antagonistic interactions among the chemicals or adaptive oxidative defense mechanisms that mitigate AOPP formation. These findings underscore the complexity of oxidative stress responses, demonstrating that both the dose and combination of chemicals critically influence the oxidative status of different organs.

The elevated levels of MDA and AOPP observed in our study underscore the significant oxidative stress induced by the combined exposure to the studied disrupters. This oxidative stress reflects an imbalance between the production of reactive oxygen species (ROS) and the body's ability to detoxify these reactive intermediates or repair the resulting damage.

To further understand the impact of TCS and BPA on the antioxidant defense system, we examined the level of the non-enzymatic antioxidant tripeptide (GSH) and the activity of the antioxidant enzyme glutathione peroxidase (GSH-Px). These actors play crucial roles in mitigating oxidative damage by neutralizing ROS and repairing oxidative damage.

In our study, we observed no significant differences in glutathione levels in the organs of animals treated with varying doses of BPA/Triclosan (TCS). Contrarily, significant increases in GSH levels were detected in the livers and kidneys of animals treated with both the lowest and highest doses of BPA, and in the ovaries of animals exposed to high BPA doses. This is intriguing when compared to the findings of **Wu et al., 2011**, where BPA in combination with nonylphenol (NP) resulted in decreased GSH levels and impaired antioxidant enzyme activity, likely due to reduced glutathione reductase (GR)

activity and diminished NADPH supply for GSH recycling. Supporting evidence from **Mourad and Khadrawy (2012)** and **Tiwari et al.(2012)** indicated that BPA exposure leads to elevated lipid peroxidation in liver and testicular tissues, suggesting oxidative stress, which could prompt an increase in GSH levels as an adaptive response to counteract oxidative damage **(Hendawy et al., 2018)**. In contrast, **Hendawy et al., 2018)** reported a decrease in GSH levels in the liver and testes with both low and high BPA doses, attributed to heightened GSH consumption for reactive oxygen species (ROS) scavenging. Our findings of increased GSH levels in specific tissues may reflect a nuanced compensatory mechanism or an initial adaptive response to oxidative stress induced by BPA, diverging from patterns observed in other studies, possibly due to differences in experimental conditions, BPA dosages, or the presence of TCS, which may influence the impact of GSH regulation.

Glutathione peroxidase (GSH-Px) plays a crucial role in cellular defense against reactive oxygen species (ROS) by catalyzing the conversion of superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2), which is subsequently reduced to water, thereby mitigating oxidative stress **(Hassan et al., 2012)**. Our study demonstrated a significant decline in GSH-Px activity in the kidney and ovary of animals treated with high doses of bisphenol A (BPA) and triclosan (TCS), whereas low doses of this combination did not result in significant changes compared to the control group. This indicates a dose-dependent reduction in GSH-Px activity due to high-dose exposure

Supporting our findings, **Chen et al., (2011)** observed that combined exposure to dibutyl phthalate (DBP) and benzo(a)pyrene (BaP) decreased GSH-Px activity in the testis, a tissue highly susceptible to ROS damage due to its high content of polyunsaturated fatty acids. The study noted that antioxidant defenses in the testis are critical to preventing ROS-induced cellular damage, and the combined exposure led to milder adverse effects than BaP alone, suggesting complex, non-additive interactions between DBP and BaP. This aligns with our observations where the combined effects of BPA and TCS on GSH-Px activity suggest neither synergistic nor additive interactions, reflecting the unpredictable nature of environmental endocrine disruptors (EDs).

Additionally, our results showed significant decreases in GSH-Px activity in the liver, kidney, and ovary of animals treated with both low and high doses of BPA. Similar

effects were observed with high-dose TCS treatment in the ovary. This is consistent with the findings of **(Hassan et al., 2012)**, who reported that a BPA dose of 50 mg/kg significantly decreased GSH-Px and GR activities in the liver, which was corroborated by reduced gene expression levels of these antioxidant enzymes. These observations underline the detrimental effects of BPA and TCS on antioxidant enzyme activities across different tissues, indicating that BPA and TCS impair the oxidative stress response in a dose-dependent manner.

The biochemical markers of oxidative stress and the altered activity of antioxidant enzymes observed in our results provide strong evidence of the oxidative damage induced by the combined exposure to TCS and BPA at least at high doses. To further substantiate these findings, it was crucial to examine the structural changes in tissues that are often a direct consequence of oxidative stress.Histopathological analysis offers a comprehensive view of tissue integrity and can reveal the extent of cellular and subcellular damage. By examining the liver, kidneys, and ovaries of the treated Female Wistar Rats, we aimed to confirm the presence of oxidative damage (**Figure 20**).

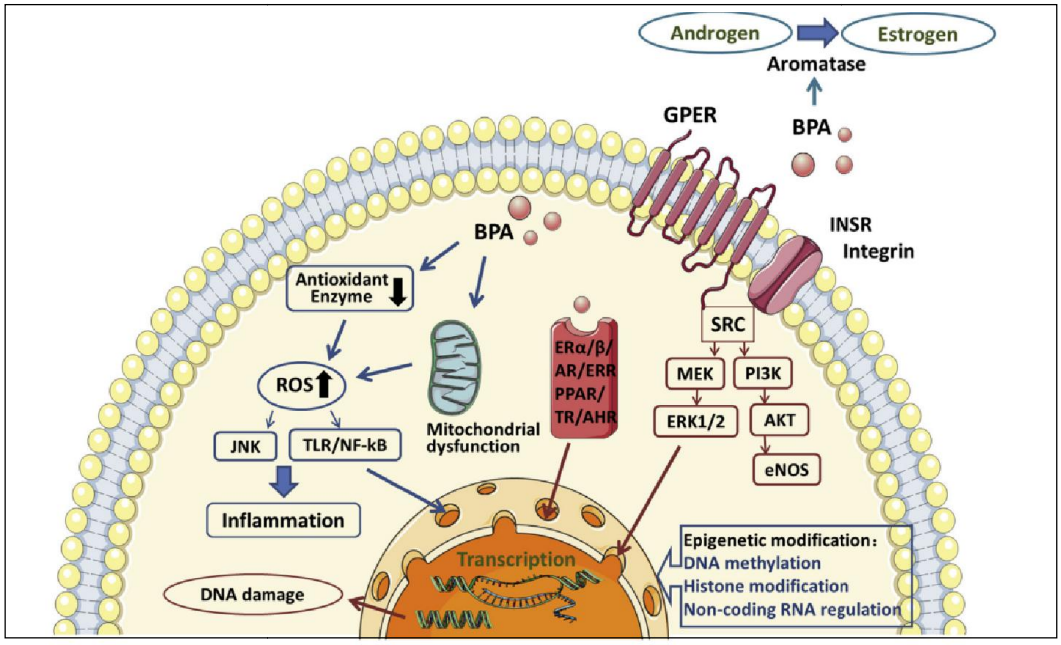

Figure 20: Schematic diagram illustrating the mechanisms of BPA toxicity **(Ma et al., 2019).**

BPA: Bisphenol A, INSR: insulin receptor, ER Estrogen receptor, AR: Androgen receptor; ERR: estrogen related receptor; PPAR: peroxisome proliferator activated receptors; TR: Thyroid

hormone receptor. AHR: Aryl hydrocarbon receptor, ROS: reactive oxygen species; JNK: c-Jun N terminal kinase TRL: toll-like receptors; SRC: proto-oncogene tyrosine-protein kinase; MEK: MAPK/ ERK kinase; ERK Extracellular regulated protein kinases; PI3K: phosphatidylinositol-3-kinases; AKT: Protein kinase B. eNOS: Endothelial nitric oxidase synthase.

The histopathological examination of the liver of animals treated with the low dose association revealed significant degenerative changes, including vascular congestion, severe bleeding, tissue damage, and fibrosis which are indicative of severe oxidative damage. Supporting our results, a study by **(Omorodion et al., 2019)**found fatty deposits and inflammatory cells in the liver tissues of rats treated orally with a mixture of 0.5 mg/kg of BPA and 0.5 mg/kg DEHP combined with pelleted rodent feed for 30 days. Cell alteration was increasingly evident in rats administered the combined BPA and DEHP therapy compared to those treated with BPA and DEHP independently. The inflammatory effect on the liver may be partly due to the detoxifying role of the liver against chemical substances in the body. Another study found vacuolation, granulations, and fatty degeneration in the liver histology of rats treated with a combination of two endocrine disruptors: polychlorinated biphenyls and diethyl phthalate in both parental and offspring (F1) generations **(Pereira et al., 2007).**

In kidney tissue, the exposure of rats to high and low doses of BPA + TCS resulted in notable alterations as the formation of hemorrhage in the kidneys, a partial degenerative lesion, an inflammatory filter, and tissue damage in the samples were observed. Similarly, in the study conducted by**Omorodion's team, (2019)**rats treated orally with a mixture of BPA and DEHP showed a reduction in Bowman capsules and glomeruli compared to the control group.

Regarding the oxidative status in the explored tissues, more marked damage was observed in the reproductive organs (ovaries) of females treated with the highest dose of BPA/TCS. The present findings indicate that exposure to low doses of BPA and TCS resulted in degenerative changes in the ovaries, including hemorrhage, vascular congestion, and inflammatory infiltrates. In contrast, the damage was more severe in the ovaries of females exposed to high doses of BPA + TCS, showing degenerative changes with vacuolation of theca cells, vascular congestion, inflammatory infiltrates, and intrastromal hemorrhage.

Concerning the extent of the oxidative status in the explored tissues, with more marked damage in the reproductive organs (ovaries) of the females treated with the highest dose of BPA/TCS.Our result indicates that the exposure of rats to low doses of BPA and TCS resulted in degenerative changes in the ovaries, which showed hemorrhage, vascular congestion, and inflammatory filter. While the injury has been deleterious in the ovaries of females exposed to high doses of BPA + TCS showing degenerative changes with vacuolation of theca cells, vascular congestion, inflammatory filter, and intrastromal hemorrhage.

Conversely, in a study by**Tassinari et al. (2021)**, male rats treated with a combination of DEHP, DBP, and BPA showed more evident testicular toxicity in the MIX group (desquamated germinal epithelium cells, enlarged cells with hyperchromatic nuclei, multinucleated cells, and intracytoplasmic vacuoles) compared to the individual chemicals. The effects on redox status were either more prominent or present only in the MIX group, confirming that the reproductive system might be more susceptible following exposure to chemical mixtures than to individual chemicals.

Our study indicates that rats watered with filtrated wastewater did not exhibit significant oxidative stress, as evidenced by biochemical markers such as MDA, AOPP and GSH levels and glutathione peroxidase activity and supported by histopathological analysis of three organs. These findings suggest that the treatment did not induce notable organ damage, likely due to the minimal presence of triclosan and Bisphenol A in the wastewater from the treatment plant. Regarding BPA removal in water treatment plants,**(Ferrer-Polonio et al., 2021)**described an almost total removal of BPA through activated sludge treatment. In their work on**.(Ruszkiewicz et al., 2017)** found that even wastewater treatment plants remove some TCS, small amounts still enter water bodies. Consequently, TCS and its harmful byproducts, such as methyl triclosan, dioxins, chlorophenols, and chloroform, are frequently found in aquatic environments, sediments, biosolids, soils, and organisms. Despite this, our results indicate that the concentrations in treated wastewater are insufficient to induce significant oxidative stress or organ damage in rats.

Conclusion

Conclusion

This study provides comprehensive insights into the subacute toxicity of combined exposure to Triclosan and Bisphenol A in Female Wistar Rats. Our findings indicate that both compounds, individually known for their endocrine-disrupting effects, established that oxidative stress is implicated in BPA and TCS-induced liver, kidney, and ovarian injury and fibrosis.

The analysis of relative organ weights revealed substantial alterations, indicating systemic stress and potential organ-specific toxicity. Glycemic variation, observed as a decrease in serum glucose levels, underscores the metabolic disruptions caused by these endocrine disruptors. Oxidative stress markers and the activity of antioxidant enzymes were significantly altered, suggesting a compromised antioxidant defense mechanism. The histopathological examination of liver, kidney, and ovarian tissues confirmed extensive oxidative damage, with observable degenerative changes, fibrosis, and vascular congestion. These findings strongly indicate that the daily administration of 100 mg of BPA/kg in combination with 10 mg of TCS/kg of female rats is harmful to the three explored organs with more severe effects on the liver and the ovary.

On the other hand, it seems that the daily exposure of rats to the treated water collected from the Guelma wastewater epuration plant did not record harmful effects to the experimental animal, as compared with the two other BPA-TCS treated groups, allowing us to suggest that BPA and TCS could be present together in these filtered wastewaters in barely detectable quantities or there is a constrictive effect when BPA interferes with TCS.

The non-synergistic nature of the interactions between these chemicals underscores the unpredictable impacts of ED mixtures, emphasizing the need for further research into their combined mechanisms of action. Therefore, clinical, and experimental studies should be conducted in the future to determine prophylactic strategies for people chronically exposed to those chemicals that affect the endocrine system and hormones. In addition, further long-term studies as dose-response relationships, including larger groups, are required to confirm and expand upon the present findings to elucidate the precise molecular mechanisms underlying the observed toxic effects and exploring potential mitigation strategies to counteract the adverse health impacts of these ubiquitous environmental contaminants.

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Appendices

Appendices 1: list of chemicals and laboratory equipment

1. Bisphenol A: [>99.0%]

- IUPAC name: 2,2-Bis (4-hydroxyphenyl) propane
- Formula: C15H16O2
- Molar mass: 228.29 g/mol
- Melting Point: 158°C
- Solubility: Ethanol, corn oil

2. Triclosan :[>99.0%]

- IUPAC name: 5-chloro-2-(2,4-dichloro phenoxy)phenol
- Formula: $C_{12}H_7Cl_3O_2$
- Molar mass: 289.5 g/mol
- Melting Point: 120 °C
- Solubility: Ethanol, corn oil

3. Reagents and products used

- Hydrogen chloride (HCl),
- Methanol, Ethanol
- Sodium chloride (NaCl), potassium chloride (KCl),
- Bovine serum albumin (BSA)
- Trichloroacetic acid (TCA)
- 5,5'dithiodis-2-nitrobenzoic (DTNB)
- GSH
- Formalin
- Distilled water
- 1-Chloro-2,4-dinitrobenzene(CDNB)
- Gornall Reagent
- Hydrogen peroxide H□O□
- Thiobarbituric acid (TBA)
- n-Butanol

- Phosphate buffered saline (PBS)
- Tompon phosphate (0.1 M PH=7.4)
- Potassium iodide (KI)
- Glacial Acetic acid
- Tris
- Ethanol
- Xylene
- Eosin
- *Mayer's reagent*[mercuric chloride (1.36 g) and potassium iodide (5.00 g)].
- Acetone

4. Laboratory equipment

- Desiccator
- Dissection kit
- SIGMA horizontal centrifuge.
- SP-UV 2005 molecular transmission spectrophotometry.
- IKA T18 basic type homogenizer.
- Laboratory water bath
- Electric balance type KERN EMB 2200-O
- Magnetic stirrer with a hot plate.
- Micropipette, spatula
- Conservation boxes
- Petri dishes
- Glassware (lumber, Erlenmeyer, crystallizer, test tube, test tube, dry tube, graduated pipette, quartz tank).
- Vortex

Appendices 2.: Calibration curves

A / Glutathione GSH calibration curve

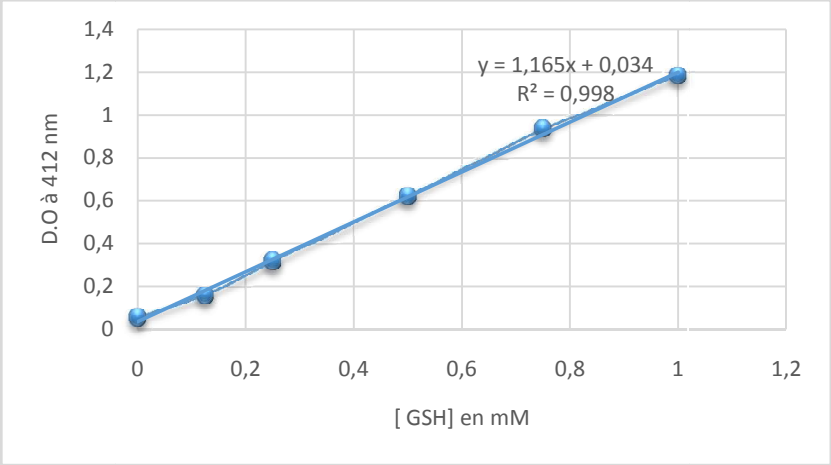

Figure 01: GSH Calibration Line

Appendices 3:Protein assay kit datasheet

Ccromatest

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TOTAL PROTEIN CE

REF 1153005	REF 1153010	REF 1153020
2 x 50 mL	4 x 100 mL	4 x 250 mL
CONTENU	**CONTENU**	**CONTENU**
Réactif R1. 2 x 50 mL	Réactif R1. 4 x 100 mL	Réactif R1. 4 x 250 mL
Standard CAL.1 x 3 mL	Standard CAL.1 x 3 mL	Standard CAL.1 x 3 mL
Uniquement pour diagnostic *in vitro*		

PROTEINES
TOTALES
Méthode Colorimétrique
POINT FINAL

PRINCIPE

Dans la réaction de Biuret, un chélate est formé entre l'ion Cu^{2+} et les liaisons peptidiques des protéines en milieu alcalin pour former un complexe violet, dont l'absorbance est mesurée par photométrie. L'intensité de la coloration produite est proportionnelle à la concentration de la protéine dans l'échantillon.[1-3]

$$Cu^{2+} + \text{Sérum protéine} \xrightarrow[25\text{-}37°C]{pH > 12} \text{Complexe Cuivre-protéine}$$

COMPOSITION DES REACTIFS

R1 **Réactif de Biuret**. Sulfate cuprique 6 mmol/L, sodium-potassium-tartrate 21 mmol/L, iodure de potassium 6 mmol/L hydroxyde de sodium 0,75 mol/L. C **R:34**

CAL **Standard Protéine.** Sérum albumine bovine 7 g/dL (70 g/L). La valeur de la concentration est traçable au Matériel Standard de Référence 927.

CONSERVATION ET STABILITE

Conserver à 2-8°C.
Tous les composants du kit sont stables jusqu'à la date de péremption indiquée sur l'étiquette. Ne pas utiliser les réactifs au-delà de cette date de péremption.
Conserver les flacons hermétiquement fermés, protégés de la lumière et hors des contaminations pendant l'usage.

Se débarrasser des réactifs s'il apparaît des signes de détérioration:
- Présence de particules et d'une turbidité.
- Absorbance du blanc réactif (A) à 540 nm > 0,150 dans une cuve de 1 cm d'épaisseur.

PREPARATION DES REACTIFS

Les réactifs et standard sont prêts à l'emploi.

ECHANTILLONS

Sérum ou plasma hépariné.
La protéine totale est stable dans le sérum et le plasma pendant 1 semaine à la température ambiante, pendant au moins pendant 1 mois s'il est réfrigéré à 2-8°C, et jusqu'à 2 mois à –20°C.

INTERFERENCES

- Lipémie (intralipides) peut affecter les résultats.
- La Bilirubine (20 mg/dL) n'interfère pas.
- L'Hémoglobine peut affecter les résultats.
- D'autres médicaments et substances peuvent interférer [3].
- Le Dextrine utilisé pour augmenter le volume du plasma dans le traitement de la pression artérielle, complexe avec le Cuivre et le tartrate formant un précipité.

MATERIEL AUXILIAIRE

- Photomètre or colorimètre capable de lire l'absorbance à 540 ± 20 nm.
- Incubateur réglée à température constante de 37°C.
- Pipettes pour mesure et distribution des réactifs et échantillons.

PROCEDURE

1. Pipeter dans les tubes à centrifuge étiquetés:

TUBES	Blanc	Echantillon	Standard CAL.
Réactif de Biuret R1.	1,0 mL	1,0 mL	1,0 mL
Echantillon	–	20 µL	–
Standard CAL.	–	–	20 µL

2. Mélanger et incuber les tubes pendant 5 minutes à 37°C.
3. Lire l'absorbance (A) des échantillons et du standard à 540 nm contre le blanc réactif.

La coloration est stable pendant au moins pendant 1 heure.

CALCULS

$$\frac{A_{Echantillon}}{A_{Standard}} \times C_{Standard} = \text{g/dL de protéine totale}$$

Les échantillons dont les concentrations sont supérieures à 12 g/dL doivent être dilués à la proportion de 1:2 avec de l'eau physiologique et testés de nouveau. Multiplier les résultats par 2.

Pour exprimer les résultats en unités SI, appliquer: g/dL x 10 = g/L.

VALEURS DE REFERENCE [4]

Sérum, plasma

Adultes	6,6 – 8,7 g/dL (66 - 87 g/L)
Prématurés	3,6 – 6,0 g/dL (36 - 60 g/L)
Nouveaux-nés	5,3 – 8,9 g/dL (53 - 89 g/L)
Grossesse	Concentration abaissée de 69 à 61 g/L

Les protéines totales sériques sont élevées de 4 à 8 g/L chez le sujet couché que chez le sujet en mouvement.

Plasma

Les protéines plasmatiques sont élevées de 2 à 4 g/L à cause de la présence du fibrinogène dans l'échantillon.

Il est recommandé que chaque laboratoire établisse sa propre plage de valeurs de référence.

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Cromatest

LINEAR

CONTROLE DE QUALITE

L'usage d'un standard pour calculer les résultats permet d'obtenir une précision indépendante du système ou de l'instrument utilisé. Pour assurer un contrôle de qualité (QC) approprié, chaque test doit inclure un ensemble de contrôles (normal et anormal), traités comme ayant des valeurs inconnues.

REF **1980005** HUMAN MULTISERA NORMAL
Evalué comme niveau normal de protéines totales.

REF **1985005** HUMAN MULTISERA ABNORMAL
Evalué comme niveau élevé de protéines totales.

Si les valeurs sont en dehors de la plage définie, vérifiez l'instrument, les réactifs et la procédure.
Chaque laboratoire doit établir ses propres plans de contrôle de qualité interne et les mesures correctives, dans le cas où les résultats des contrôles sont en hors des tolérances acceptables.

INTERPRETATION CLINIQUE

Le sérum contient des protéines solubles, qui circulent dans les liquides extracellulaires et intracellulaires, et utilisées comme marqueurs pour aider le diagnostic clinique. Les principaux tests diagnostic sont ceux qui mesurent les protéines totales sériques et l'albumine sérique.
Collectivement, toutes les protéines sériques l'albumine y comprise sont principalement impliquées dans le maintient de la distribution normale de l'eau entre les tissus et le sang, et sont responsables du maintient de la pression osmotique du plasma, et transportent beaucoup de substances ainsi que les macromolécules.
Hyper protéinémie ou hyper albuminémie survient généralement dans le myélome multiple à cause d'une augmentation des immunoglobulines monoclonales, dans la déshydratation, et les pertes excessives d'eau telles dans les vomissements et les diarrhées sévères, dans la maladie d'Addison ou dans l'acidose diabétique. L'*hémoconcentration*, diminution du volume d'eau plasmatique, est reflétée par une relative hyper protéinémie puisque les concentrations de l'ensemble de protéines sont élevées au même degré.
Hypo protéinémie ou hypo albuminémie survient généralement dans l'oedème, la malnutrition, le syndrome néphrotique, la malabsorption et la cirrhose sévère de foie. Puisque quantitativement l'albumine est la protéine la plus importante du sérum, des petites baisses de cette seule protéine peuvent aussi causer une hypo protéinémie.

CARACTERISTIQUES ANALYTIQUES

- ***Limite de détection :*** 0,31 g/dL
- ***Linéarité :*** Jusqu'à 12 g/dL
- ***Précision:***

g/dL	Intra-série		Inter-série	
Moyenne	4,33	8,99	4,33	8,99
SD	0,05	0,14	0,07	0,24
CV%	1,20	1,59	1,58	2,65
N	10	10	10	10

- ***Sensibilité:*** 0.05 A / g/dL de protéines.
- ***Corrélation.*** Ce test (y) a été comparé avec une méthode commerciale similaire (x). Les résultats suivants ont été obtenus:
  N = 64 r = 0,95 y = 0,99x + 0,20

Ces caractéristiques analytiques ont été obtenues en utilisant des équipements automatiques. Les résultats peuvent varier en fonction de l'équipement.

NOTES

1. Cette méthode peut être utilisée avec n'importe quel appareil. Toute application sur un appareil devrait être validée par une démonstration de la concordance des résultats avec les caractéristiques analytiques de la méthode. Il est recommandé de valider périodiquement l'appareil. Veuillez contacter le distributeur pour toutes questions relatives à l'application de la méthode.
2. Le diagnostic clinique ne devrait pas se limiter sur les seuls résultats du test, mais intégrer corrélativement les données cliniques et de laboratoire.

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