DEVELOPMENT OF GREENER ULTRASOUND AND MICROWAVE BASED SAMPLE PREPARATION METHODS FOR SPECTROSCOPIC DETERMINATION OF HEAVY METALS IN PET FOOD SAMPLES

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DECLARATION

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Development of greener ultrasound and microwave-based sample preparation methods for spectroscopic determination of heavy metals in pet food samples

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KEY TERMS:

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PUBLICATION(S)

This dissertation consists of one manuscript submitted and another one in preparation:

1. **Nomatter Ncube**, Yolanda Tancu, & Nomvano Mketo. *A greener, rapid, and accurate microwave-assisted hydrogen peroxide digestion method for ICP-OES determination of heavy metals in pet food samples,* Journal of Food Composition and Analysis 131 (2024) 106201. (**PUBLISHED**).
2. **Nomatter Ncube**, Maxwell Thatyana, Yolanda Tancu & Nomvano Mketo. *Quantitative analysis and health risk assessment of selected heavy metals in pet food samples using ultrasound assisted hydrogen peroxide extraction followed by ICP-OES analysis.* (**UNDER REVIEW**).

ABSTRACT

Contamination of pet food with heavy metals is a crucial problem worldwide and it results in adverse health effects on pets, massive recall of the product, damaging of the brand name and financial loss. Therefore, researchers have been constantly monitoring heavy metals in pet food samples using spectroscopic techniques especially inductively coupled plasma- optical emission (ICP-OES). However, food matrices cannot be directly introduced into the standard ICP-OES there is always a need for a sample preparation step. Mostly reported wet chemistry sample preparation method have had limitations due to large use of concentrated acids. Thus, development of new methods, that is; minimizing use of concentrated acids, reduction of energy consumption and utilizing reusable materials are the future trends for principles of green chemistry. Therefore, the current study aimed at developing and validating greener microwave and ultrasound-based sample preparation methods for determination of cadmium (Cd), arsenic (As), lead (Pb), tin (Sn), and chromium (Cr) prior to ICP-OES analysis. The optimum conditions (200 °C, 40 mins, 0.5g, and 5 mol/L for temperature, digestion time, sample mass and H_2O_2 concentration, respectively) for microwave- assisted hydrogen peroxide digestion (MW-AHPD) were investigated using multivariate tools, certified reference material wheat flour (FAP80467) and fish (ERMBB422). The MW-AHPD demonstrated excellent accuracy (96-98%), reproducibility (≤ 2.1%) and method detection limits of 0.0675 to 0.3765 µg/g. Application in real pet food samples made from maize, rice, vegetables, fish, and wheat reported concentration levels of Cd, Pb, As, Cr and Sn ranged between 11.2-22.6, 6.4-11.9, 3.44-13.4, 0.44-2.98 and 0.18-0.98 µg/g, respectively.

Furthermore, the optimum conditions for ultrasound assisted hydrogen peroxide extraction (UA-HPE) were: 80 °C, 60 mins, 0.5g, 5 mol/L, for sonication temperature, extraction time, sample mass, and H_2O_2 concentration, respectively. The UA-HPE method was accuracy (>95%), precise (≤ 1.9 %) with acceptable method detection limits (0.3498 and 0.49 µg/g). The reported concentration levels by UA-HPE were 0.86-11.34, 4.50-11.45, 2.61-12.5, <DL-7.94, and <DL-1.04 µg/g for Cd, Pb, As, Cr and Sn, respectively. Both methods (UA-HPE and MW-AHPD) were assessed for greenness using the AGREEPrep metric tool and had similar scores of 0.74 (UA-HPE) and 0.76 (MW-AHPD), confirming the greenness. It is worthy to indicate that maximum concentration limits for Cd, Pb, As, Cr and Sn in pet food samples are: (10, 10, 10, 12.5 and 200 µg/g respectively) as reported by South African regulators. However, the current study showed

that Cd (11.2-22.6 µg/g), Pb (11.45-11.9 µg/g) and As (12.5-13.4 µg/g) were above the maximum tolerable limits, for the fish and wheat-based pet food samples. Therefore, these results imply that South African pets are at risk from consuming wheat and fish-based pet foods. More studies on health risk assessment are required to further confirm the findings.

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LIST OF ABBREVIATIONS

MW-AHPD	Microwave assisted hydrogen peroxide digestion.
UA-HPE	Ultrasound-assisted hydrogen peroxide extraction
UAD	Ultrasound-assisted digestion
PHWE	Pressurized Hot Water Extraction
MAE	Microwave-assisted extraction
MAD	Microwave-assisted digestion
MIC	Microwave Induced Combustion
IDL	Instrument Detection Limit
ISO	International Organization for Standardization
LOD	Limit of Detection
LOQ	Limit of Quantification
MDL	Method Detection Limit
QC	Quality Control
PFI	Pet Food Industry
RSD	Relative Standard Deviation
ICP-OES	Inductively Coupled Plasma-Optical Emission Spectrometry
FAAS	Flame Atomic Absorption Spectroscopy
GFAAS	Graphite Atomic Absorption Spectroscopy
CCD	Central Composite Design

CHAPTER I (INTRODUCTION)

PREAMBLE

Chapter one provides background information on pet food, heavy metals, and their toxic health effects on pets. The chapter highlights problem statement, hypothesis, justification, and motivation for conducting this study. Furthermore, the main goal and specific objectives of this study are listed, together with the outline of the dissertation.

1.1 BACKGROUND

Pet food is produced for domesticated animals to meet all nutritional needs and as complementary treats (Schleicher, 2019). The most common pets found in South African households are dogs, cats, birds, and horses. The main raw materials for pet food include but are not limited to plant fats, cereals, grains, minerals, preservatives, flavorings, vitamins, seafood, meat, and animal derivates (Van Rooijen, 2013). Pet foods can be grouped differently, that is (i.e.), dry foods made by baking or extruding, wet food made by cooking and semi-moist food made by baking or extruding with final moisture of 60 to 65 % (Leiva, 2019; Riaz, 2011; Rokey, 2010).

Pet food types usually require specific type of packaging. For an example, wet foods are usually packaged in airtight cans, trays, and pouches, on the other hand semi-moist and dry pet foods are usually packaged in airtight plastic bags, skillets or tubs and placed in cartoon boxes (Schleicher, 2019). Primarily, packaging should be airtight to preserve shelf life of the product as air encourages microbiological organism’s growth (Haverkamp, 2020). Throughout all the processing steps, several measures are implemented to make sure the products are made of safe and quality ingredients and are produced under hygienic condition, with no introduction of hazards (physical, chemical, and biological) which can contaminate the product which in turn can affect the consumer (Carrión, 2023; Montegiove, 2021). Majority of all pet foods are stored at ambient temperature hence they are transported in covered, lockable trucks at ambient temperatures to customers and distributors (Van Rooijen, 2013). The main ingredients (maize, flour, nuts, meat, and fish) in pet food formulation are susceptible to contamination by heavy metals (Høgåsen et al., 2016). Heavy metals occur in nature and are not biodegradable. Hence they can easily accumulate in the environment to lethal levels which can affect living organisms such as animals, fish, crops, and humans (Squadrone et al., 2017). Since the main ingredients used in pet food formulation are susceptible to heavy metal contamination, use of contaminated ingredient/s can result in contamination of the whole batch of the product (Kazimierska et al., 2020).

1.1.1 Heavy metals

Heavy metals are toxic metals and metalloids that can cause severe pollution and toxicity. A heavy metal becomes a contaminant when it is found where it is not desirable. Heavy metals or metalloids include Cu, Hg, Zn, Ag, Cd, Se, Ni and Pb (Pandey & Sharma, 2014). Among these, Cd, Pb, As, Cr, and Sn are the most toxic known heavy metals to pets, hence were selected as analytes of interest for the study. The most common sources of contamination of heavy metals are water sources and soil (Gunalan *et al.,* 2018). As shown in **Fig.1. 1**, water sources, soil and air can get contaminated with heavy metals released from industrial processes such as waste disposal, pulp and paper manufacturing, mining, metal plating, paint, and pigments manufacturing (Sandeep, 2019). This contamination can reach areas of crops (wheat, maize, rice, legumes, etc.) and livestock farming (cattle, pigs, poultry, fish, etc.) which can have their products and by products used in pet food formulation. Use of contaminated ingredients in pet food formulation may result in high levels of heavy metals.

Continuous ingestion of pet food with high levels of heavy metals is associated with heavy metal poisoning which may lead to both acute and chronic health effects in pets. Cadmium poisoning in pets affects all major organs, mainly the kidneys and liver leading to degeneration of renal tubule, renal atrophy, and renal functional failure (Council, 2006). Lead poisoning causes acute health effects, namely vomiting, anorexia, and diarrhoea (Council, 2006; Høgåsen et al., 2016). Arsenic poisoning affects the vascular system of pets, once arsenic enters the body it travels to the main organs, causing bleeding and swelling. Signs of acute arsenic poisoning are diarrhoea, vomiting, weakness, staggering, low body temperature, collapsing, and death (Garland, 2020; Gupta, 2018; Schmid *et al.,* 2021). Additionally, chromium exists in its trivalent and hexavalent forms; the hexavalent form is the most toxic to pets (Esposito *et al.,* 2019; Macías-Montes *et al.,* 2021). There is limited evidence to support toxicity of chromium in pets, however chronic exposure to Cr leads to damage of the circulatory system, nerve tissues, kidney, and liver failure

(Macías-Montes *et al.*, 2021). Tin is relatively low in toxicity, however if consumed in high concentration it may affect kidneys, liver, and pancreas (Esposito *et al.*, 2019).

Consequently, determination and quantification of heavy metals in pet food must be performed accordingly to monitor the levels of heavy metals to prevent intoxication in pets. Nevertheless, literature has shown that little studies have been done on development of efficient methods for quantification of heavy metal pollutants in pet foods (Abd-Elhakim *et al.*, 2016; Pereira *et al.*, 2018; Zafalon *et al.*, 2021). Therefore, there is a need to keep continuously improving the current determination methods to achieve a specific, sensitive, and simple methodology.

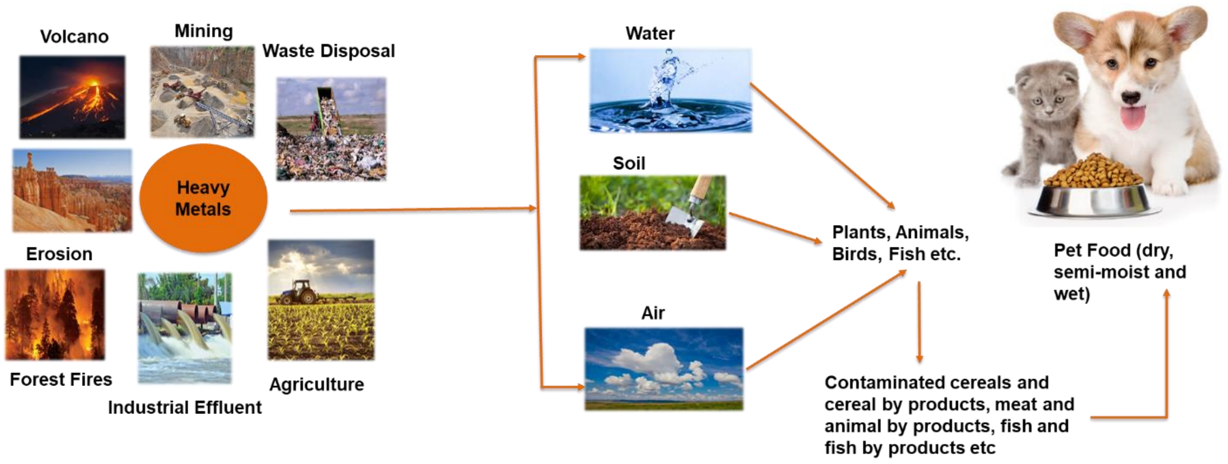

Figure 1. 1: Sources of heavy metals and their occurrence in pet food chain.

1.1.2 Determination of heavy metals in pet food

Several analytical techniques have been used in determination of metal ions in several food sample matrices. These techniques include flame atomic absorption spectrometry (AAS), graphite furnace atomic absorption spectrometry (GFAAS), inductively coupled plasma spectroscopy (ICP-MS), and inductively coupled plasma optical emission spectroscopy (ICP-OES) (Acar *et al.*, 2016; Muller *et al.*, 2016; Šelih et al., 2014; Shukor *et al.*, 2015). FAAS is the easily accessible technique, found in most laboratories, owing to its low operational costs and good performance, however its big limitation is its mono-elemental detection and limited linearity (Gonzálvez & De La Guardia, 2013). As a result, in the past decade ICP-OES has become its biggest competitor in elemental analysis, owing to its multi-elemental detection capabilities (Bulska & Wagner, 2016; Douvris et al., 2023). The biggest competitor for ICP-OES in elemental analysis is ICP-MS. ICP-OES has extremely lower detection limits (1000x). Biggest limitations for both ICP-OES and ICP-MS are their high operational costs (Agatemor & Beauchemin, 2011). Analysis of metal ions using these techniques requires sample preparation prior to analysis by these techniques (Ahmat Mohamed, 2020; Wojnowski et al., 2022a). These sample preparation techniques can be grouped into extraction (Alves *et al.,* 2023b; Costa *et al.,* 2020a; Curti *et al.,* 2021; Gamela *et al.,* 2020), wet chemistry (Acar *et al.,* 2016; Ahmat Mohamed, 2020; Alhagri & Albeshry, 2023; Gianluigi Maria Lo Dico, 2018) , and combustion (Barin, 2014b; Crizel, 2015b; Muller, 2013; Picoloto, 2016). Extraction methods involve separating analytes of interest from the sample matrix using a solvent such as acids i.e., nitric acid, hydrogen peroxide, and hypochlorous acid. In wet chemistry methods, samples are completely decomposed using solvents such as acids usually at high temperatures. Combustion methods involve decomposing samples in a vessel in the presence of oxygen at high temperatures. The selection of the sample preparation method depends on various factors including sample matrix, targeted analyte, budget, and the detection technique of interest, just to name a few (Szymczycha-Madeja, 2014). However, all the sample preparation methods also have their demerits and merits. The biggest demerit of these sample preparation methods is that they are not in compliance and harmony with Green Analytical Chemistry (GAC). To bridge this gap, there has not only been an increase in development of greener sample preparation methods but also an increase in development of metric tools to assess the greenness of sample preparation methods.

1.1.3 Assessment of greenness of sample preparation methods

In the last decade, there has been a widespread interest in green chemistry due its ability to harness chemical innovation to simultaneously meet economic and environmental goals *(*Bizzi *et al.,* 2017). Green analytical chemistry (GAC) concept was first introduced in 1998 by P. Anastas (Armenta, 2008). It is anchored on 12 principles, namely prevention, atom economy, less hazardous chemical syntheses, designing safer chemicals, use of safer solvents and auxiliaries, design for energy efficiency, use of renewable feed stocks, reduce derivatives, catalysis, design for degradation, real-time analysis for pollution prevention and inherently safer chemistry for accident prevention (Wojnowski *et al.,* 2022). Green analytical chemistry concept aims at reducing or eliminating toxic chemicals from an analytical process to improve environmental friendliness without compromising the efficiency of the process (Armenta, 2008). The first tool for assessment of greenness of an analytical method, the analytical eco-scale tool, and was introduced a decade ago as a need for analytical methods to be compliant with green analytical chemistry (GAC). Several tools have since been reported in literature, AGREEprep (Wojnowski *et al.,* 2022), National Environmental Methods Index (NEMI (Mohamed, 2020), Eco-scale (Gałuszka, 2012), Green Analytical Procedure Index (GAPI) (Attia, 2023), and AGREE (Pena-Pereira, 2022). AGREEprep was recently introduced in the form of 10 principles, to address the exclusion gap created by GAC, which excludes sample preparation step from green analytical chemistry (Pena-Pereira, 2022). Although AGREE provided a simpler and better method for assessment of greenness of an analytical method, it did not measure the environmental impact of the sample preparation method (Pena-Pereira, 2022) hence the introduction of AGREEprep (Wojnowski et al., 2022a) in 2022 to cover this gap. Consequently, this work made use of AGREEprep as it is a newly developed tool for assessment of greenness of a sample preparation method which is more advanced and specifically designed to cover gaps which have been identified in the previously developed tools.

1.2 PROBLEM STATEMENT

Several sample preparation methods have been developed for spectroscopic determination of heavy metals in food matrices. The well reported methods include microwave acid assisted digestion (Acar *et al.*, 2016; Ahmat Mohamed, 2020), pressurized hot water assisted extraction (Plaza & Turner, 2015), ultrasound acid assisted extraction (Curti *et al.*, 2021; Gamela et al., 2020), and microwave-induced combustion (Muller, 2013). The biggest challenge with acid assisted methods is that they do not support green analytical chemistry (GAC) (Armenta, 2008; Bizzi, 2017a; Nowak, 2021), they use concentrated acids which produce hazardous residues during extraction and digestion, while combustion methods make use of expensive oxygen and quartz vessels (Barin, 2014b). Therefore, there is an urgent need to develop more reliable, cost-effective, and greener sample preparation methods which are in harmony and compliance with GAC for monitoring of heavy metals in pet food samples.

1.3 OBJECTIVE OF STUDY

1.3.1 Main Objective

The aim of the study is to develop rapid, greener, and effective ultrasound and microwave-based sample preparation methods for the determination of heavy metals in various South African pet food samples using inductively coupled plasma optical emission spectroscopy (ICP-OES).

1.3.2 Specific objectives

The specific objectives of the study are:

I. *To develop microwave-assisted hydrogen peroxide digestion method (MW-AHPD) followed by inductively coupled plasma-optical emission spectroscopy (ICP-OES) analysis for determination of metal ions in various South African pet foods.*
    - The most influential parameters (sample amount, microwave temperature, digestion time and hydrogen peroxide concentration) of the MW-AHPD were optimised using multivariate mathematical tools.
    - Analytical figures of merit such as method detection limits, accuracy, linearity, and precision were investigated by using certified reference materials.
    - The optimum parameters of MW-AHPD were then applied in real pet food samples.
    - The greenness of the MW-AHPD was assessed by using AGREEprep metric tool.

II. *To develop ultrasound assisted extraction (UAE) using diluted hydrogen peroxide for extraction of metal ions in various South African pet foods prior to inductively coupled plasma-optical emission spectroscopy (ICP-OES) analysis.*
    - The parameters (ultrasound temperature, extraction time, sample mass and hydrogen peroxide concentration) affecting UA-HPE were optimised using multivariate mathematical tools.
    - Analytical features such as method detection limits, accuracy and precision of the method were investigated by using certified reference materials.
    - The optimized UA-HPE was then applied in real pet food samples prior to ICP-OES analysis.
    - Assessment of greenness of the UA-HPE using AGREEprep metric tool.

III. To compare MW-AHPD and UA-HPE methods in terms of their merits and demerits.

1.4 JUSTIFICATION OF STUDY

Worldwide reports of heavy metal contamination of food has become a global issue leading to an increase in development of sample preparation methods for the determination of these metal ions in food (Gunalan et al., 2019). Among the preferred sample preparation methods are microwave acid digestion (Alhagri & Albeshry, 2023; Bizzi, 2017b), ultrasound acid extraction (Alves *et al.*, 2023a; Costa *et al.*, 2020b), and microwave-induced combustions (Barin, 2014a; Crizel, 2015a; Mesko, 2010), just to name a few. The biggest challenge of these classical sample preparation is that they do not support green analytical chemistry, as they use concentrated solvents, which are corrosive, generate toxic residues, and possible explosion (Wojnowski et al., 2022b) and are costly. To overcome this challenge, there have been attempts in development of sample preparation methods that are greener, with emphasis on the use of safer, less toxic solvents. Therefore, the study demonstrates the development of greener sample preparation methods which comply with GAC.

1.5 HYPOTHESIS

Sample preparation methods such as microwave assisted digestion and ultrasound assisted extraction methods will enable quantitative separation of heavy metals in different pet food sample matrices prior to ICP-OES analysis.

1.6 DISSERTATION OUTLINE

- **Chapter 1** presents the introduction or background on pet food heavy metals, and it also covers statement of problems, hypothesis, objectives, justification, and motivation of this study.

- **Chapter 2** provides detailed literature review on various techniques for sample preparation and quantification of heavy metals in different sample matrices. An overview of different types of sample preparation techniques used in food samples, namely: digestion, combustion, and extraction and their merits**.** Chapter two also critically reviews the optimization procedures (univariate vs multivariate) used to investigate the most influential parameters. The chapter also goes further on to highlight the principles of green chemistry and it should be assessed for any newly developed method.

- **Chapter 3** is all about the detection techniques mostly reported for determination of heavy metals. This also gives an understanding of the basic principles, demerits, and merits for each instrument.

- **Chapter 4** reports on the determination of heavy metals in pet food using inductively coupled plasma-optical emission spectroscopy (ICP-OES) after microwave assisted hydrogen peroxide digestion (MW-AHPD) and ultrasound assisted hydrogen peroxide extraction (UA-HPE). It gives a detailed experimental procedure for MW-AHPD and UA-HPE method and multivariate optimization of the different parameters (microwave and ultrasound temperature, digestion/ extraction time, concentration of hydrogen peroxide, and sample mass) of this method prior to application in pet food samples. It goes further to give a scope on the validation of the methods and assessment of greenness of the MW-AHPD and UA-HPE using AGREEprep tool. Lastly it presents and discusses the results obtained.
- **Chapter 5** presents the summary of the findings of this study, comparison of the two sample preparation methods (MW-AHPD and UA-HPE), and the conclusion drawn from the results obtained as well as future recommendations.

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CHAPTER II (LITERATURE REVIEW)

PREAMBLE

Chapter two provides detailed literature review on recent developments within 2013-2023 period and applications of various techniques for sample preparation and quantification of heavy metals in different food sample matrices. An overview of wet chemistry sample preparation methods such as: ultrasound acid assisted digestion (UAD), microwave acid assisted digestion (MAD), block acid digestion as well as extraction sample preparation methods namely: pressurized hot water extraction (PHWE), microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), and combustion sample preparation methods i.e. induced combustion (MIC), and oxygen flask combustion are discussed. The review emphasizes extraction and digestion acid solvents, element analytes, and detailed description of the basic principles, limitations, and merits of each sample preparation method. Furthermore, a review of application of greenness assessment tools such as Eco-Scale (ESA), Green Analytical Procedure Index (GAPI), NEM and AGREE (Kowtharapu, 2023), and AGREEprep and optimization tools (multivariate and univariate) is outlined together with their merits and limits.

2.1 DETERMINATION OF HEAVY METALS IN FOOD SAMPLES

A lot of research studies have been carried out on determination and quantification heavy metals in different food matrices (Aiko, 2015; AlFaris, 2020; Khaneghah *et al*., 2018) using different extraction methods (Gu, 2013; Zou *et al.,* 2013) and quantification techniques (Crizel, 2015; Muller, 2013; Pereira, 2013; Picoloto, 2016). The maximum concentration limits for Cd, Pb, As, Cr and Sn in pet food are 10, 10, 10, 12.5 and 200 μg/g, respectively as reported by South African pet food regulatory standard (ACT 36 of 1947). Determination and quantification of heavy metals requires extensive sample preparation prior to analysis, to isolate the trace target analyte and eliminate any possible sample matrix interferences because majority of food sample matrices are complex and may cause serious matrix effects if not removed prior to analysis (Agatemor & Beauchemin, 2011). Sample preparation is the most crucial and challenging step prior detection and quantification of heavy metals (Wilschefski & Baxter, 2019). It is time consuming (70%), depending on the matrix type involved, which can complicate the process for further analysis (Vaghel *et al.,* 2016). Errors may occur in this step, which may affect the results outcome of analysis. Moreover, the choice of the sample preparation technique is purely dependent on the state and the type of the sample matrix, and the detection technique (Szymczycha-Madeja, 2014). A good choice of sample preparation saves time and gives accurate results. A plethora of extraction sample preparation techniques have been utilized before for preparation of food samples prior to analysis as shown in **Fig. 2.1**. Traditional sample preparation methods for determination of heavy metals in food samples can be classified into:

i. *Wet chemistry methods*.

Wet chemistry methods involve use of oxidizing acid digestion reagents to separate analytes from the sample matrix at high temperatures, completely decomposing the sample matrix and include: microwave assisted digestion (MAD) (Acar *et al.,* 2016; Mketo *et al.,* 2015; Muller *et al.,* 2016), ultrasound assisted digestion (UAD) (Mayotha, 2023; Siriangkhawut, 2017), and block acid digestion (BAD) (Ávila, 2017).

ii. *Extraction methods*

Extraction methods involve use of oxidizing acid extraction reagents to separate the analytes from sample matrix at medium temperatures, usually the sample matrix is not decomposed and include: UAE (Gu, 2013; Zou *et al.,* 2013), MAE (Blicharska, 2015; Dahmoune *et al.,* 2015; Ekezie, 2017; Llorent-Martínez, 2014; Mullapudi *et al.,* 2019; Mullapudi, 2019), and PHWE (Zwane, 2023).

iii. *Combustion methods*

Combustion methods involve complete decomposition of the sample matrix in a vessel in the presence of oxygen at extremely high temperature (~ 500 °C). (Zhou, 2016) and include: microwave induced combustion (MIC) (Crizel, 2015; Mesko, 2010; Muller, 2013; Pereira, 2013; Picoloto, 2016) and oxygen flask combustion.

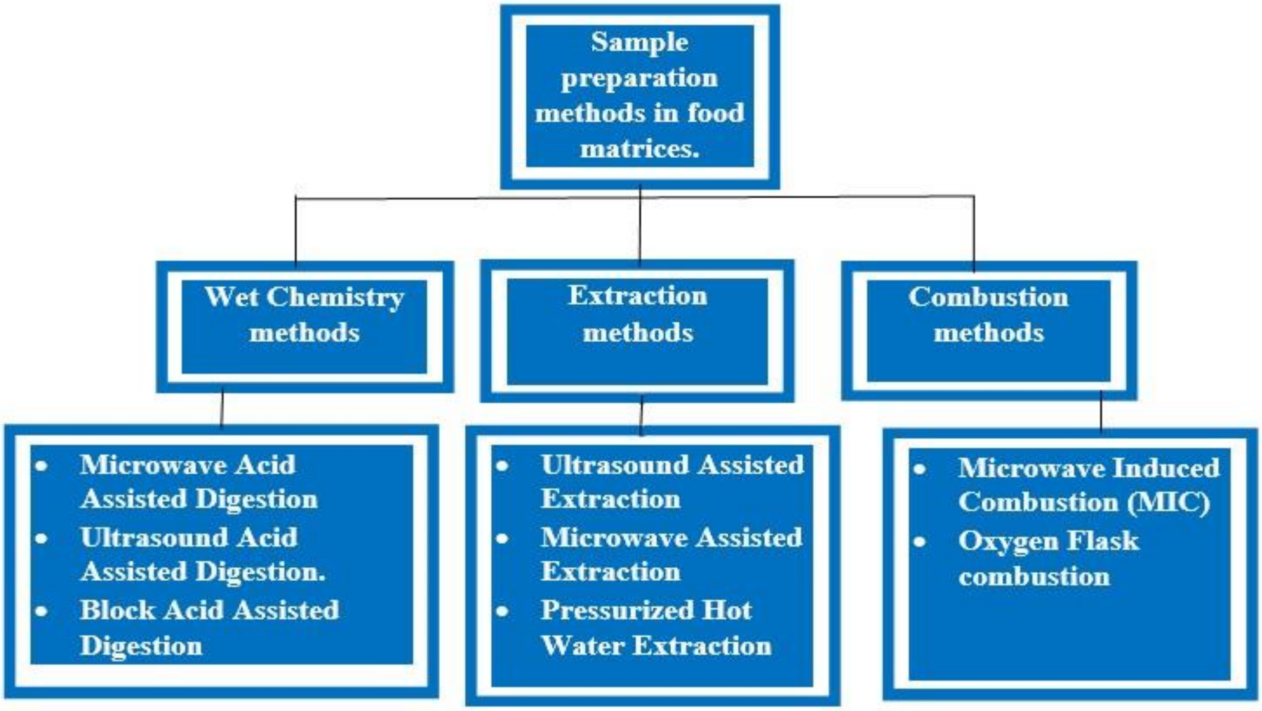

Figure 2.1: Sample preparation methods applied in food samples.

2.1.1 Wet Chemistry

In wet chemistry food samples are digested by mixing them with oxidizing acidic reagents in a vessel and heating the mixture to break the organic compounds in the food sample matrix (El Hosry *et al.*, 2023). Over the past decade, in efforts to reduce sample preparation time, sample losses by volatilization, and studies carried out at lower temperatures; wet chemistry-based sample preparation methods have gained interest over other sample preparation methods such as combustion. High temperatures may lead to loss of reagents, thus resulting in poor digestion yields Xia *et al.*, 2019. Wet digestion is usually performed in closed systems, such as microwaves which can minimize volatilization of analytes of interest. Additionally, wet digestion uses moderate temperatures (< 200 °C) compared to combustion temperatures (~ 500 °C) (Barin, *et al.*, 2014). Moderate digestion temperatures of wet digestion methods have proved to be necessary in digestion of volatile elements such as arsenic, selenium, and mercury in food samples (El Hosry *et al.*, 2023; Ferreira *et al.*, 2015). At elevated temperatures, these elements may be converted to volatiles species which may partially or completely evaporate from the sample (Ferreira *et al.*, 2015). A variety of wet digestion techniques have been developed from ambient to high pressure wet digestion in ultrasonic bath (ultrasound assisted digestion) to digestion in microwaves (microwave assisted digestion) (*Acar et al.*, 2016, Mayotha *et al.*, 2023, Zou *et al.*, 2013).

Microwave-assisted digestion (MAD)

Microwave assisted digestion uses microwave energy as source of heat for digestion of sample in presence of an oxidizing acid solvent. Microwave energy has been used as source of heat in analytical techniques since the late 1970s and it was mostly utilized in acid digestions (Abu-Smra et al., 1975). The first reported microwave-based sample preparation method was developed by Ganziler and colleagues (Ganziler *et al.*, 1986b; Ganziler, 1986a) for extraction of crude fat, gossypol, and pesticides from food and soil using domestic equipment. Ever since then, microwaves specifically for sample digestion have been developed, together with high chemical, pressure, and temperature resistant digestion vessels (polytetrafluoroethylene (PTFE)). PTFE vessels allow performance of enclosed digestion of samples at high pressure and temperatures, using strong oxidizing acids (El Hosry *et al.,* 2023; Gabano & Ravera, 2022). The main advantage of using microwave energy in digestion is that, it disrupts the weak hydrogen bonds promoted by the dipole rotation of the molecules (Kaufmann & Christen, 2002). Furthermore, the rotation rises from constant back and forth rotation of molecules, hence this constant rotation of the molecules eventually breaks the weak hydrogen bonds in molecules. Additionally, as shown in **Fig. 2.2,** unlike traditional solvent digestion techniques , microwave heating heats the entire sample-solvent mixture simultaneously then the heat is transferred directly and instantaneously to the sample-solvent mixture independently of their position in the PTFE vessel (Kaufmann & Christen, 2002). In traditional solvent digestion techniques, such as digestion bombs, the heating of the sample-solvent mixture takes place indirectly via the heated outer vessel, the heat comes from the outside of the vessel and goes into the sample-solvent mixture by convention currents (Barin, *et al.,* 2014). However, the limitations of MAD are time required for cooling of digestion vessels before they can be opened: this can take up to hours depending on the type of microwave. Another limitation of MAD is the possibility of cracking and explosion of digestion vessels under high pressures and temperatures of the microwave. In terms of merits and limitations, the biggest competitor for MAD is ultrasound assisted digestion (UAD).

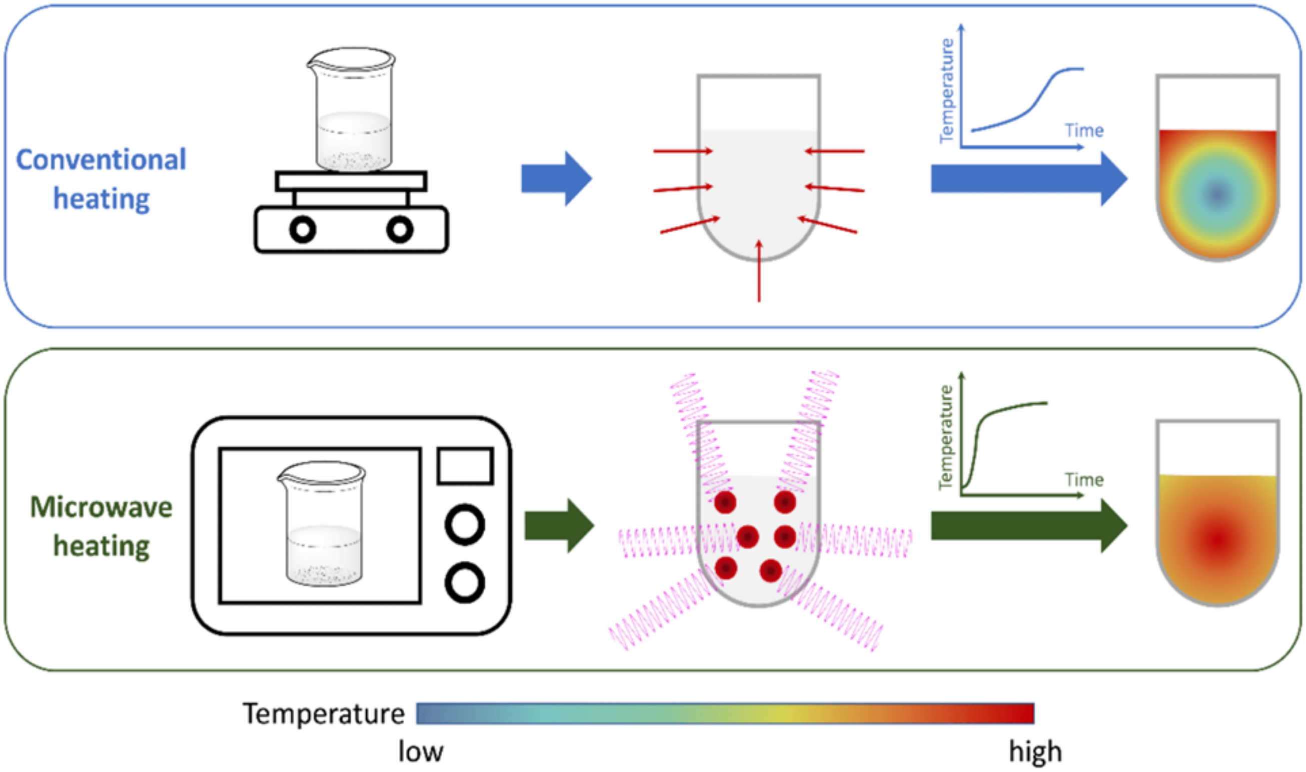

Figure 2.2: A schematic diagram showing the difference between traditional conductive heating and microwave heating (Gabano & Ravera, 2022).

Ultrasound-assisted digestion (UAD)

Ultrasound assisted digestion is utilized for digestion of target analytes in solid sample matrices assisted by oxidizing acid reagents. The UAD method is subdivided into direct and indirect method. In the direct UAD, ultrasonic radiation is applied to the solvent-sample mixture through a probe immersed in the mixture, whereas in indirect UAD, ultrasonic radiation is applied to the solvent-sample mixture via an ultrasonic water bath (Weggler 2020). It is based on physiochemical principle of acoustic cavitation due to propagation of ultrasonic waves which causes the formation, expansion, compression, and explosion of bubbles in a liquid medium (Carreira-Casais *et al.*, 2021). The UAD method is preferred over classical digestion methods because of the larger contact surface area between the solvent and the solid sample matrix, owing to the direct contact between ultrasonic waves and the analyte particles (Wiyarno *et al.*, 2010). As shown in **Fig. 2.3.** each bubble goes through a cycle which consists of different phases namely: a full wave cycle which holds expansion and compression phase (Carreira-Casais *et al.*, 2021).

During the expansion phase, also known as the rarefaction phase, gas diffuses into the bubbles because the external pressure of the bubbles is higher than the internal pressure. While during the compression phase, gas diffuses out of the bubble due to internal pressure being higher than external pressure (Carreira-Casais *et al.*, 2021). As shown in **Fig. 2.4**. The UAD is the second most reported sample preparation method in wet digestion, owing to its merits. The UAD method is considered as one of green sample preparation techniques as it uses relatively small amounts of digestion solvents, thereby generating less residual waste (Barros *et al.*, 2013). Like MAD it is rapid and confers high extraction yields. However, unlike MAD, UAD instrument (ultrasonic instrument) consumes less energy compared to microwave used in MAD. A wet digestion method which was very popular in food matrices more than a decade ago before advances in UAD and MAD it was block acid digestion (Ávila, 2017).

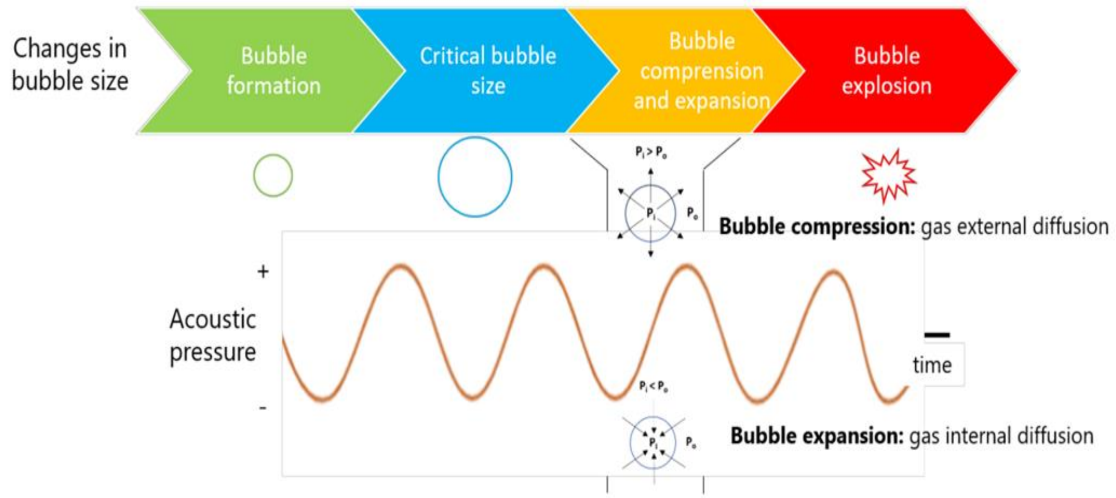

Figure 2.3: A schematic diagram of bubble growth cycle during ultrasonication (Carreira-Casais et al., 2021)

Block acid digestion (BAD)

Block acid digestion uses a digestion block to digest samples at high temperature, usually in the presence of an acidic solvent. Major limitations of block acid digestion are that it is associated with airborne contamination and poor volatile compound recovery (Ávila *et al.*, 2017). Digestion block is reported in digestion of food samples, however as shown in **Fig. 2.4** its application in wet digestion of food sample matrices has greatly declined due to introduction of better wet digestion methods such as MAD which offer advantages over this method. It is however still being reported in digestion of some food samples (Ávila *et al.*, 2017; Barea-Sepúlveda *et al.*, 2021). The biggest advantages of block digestion are its instrument simplicity and low operational costs. It is worth noting that block acid digestion is still widely reported (Do Nascimento *et al.*, 2015; Idera *et al.*, 2014; Rizwan *et al.*, 2021) in digestion of soil samples. This may be because soil samples are more challenging to digest using methods like UAD because of the silicate minerals such as quartz found in the soil. However, block acid digestion uses the same principle for digestion as MAD and UAD, using an oxidizing acid solvent under heat to separate metal analytes from the sample matrix (Barea-Sepúlveda *et al.*, 2021).

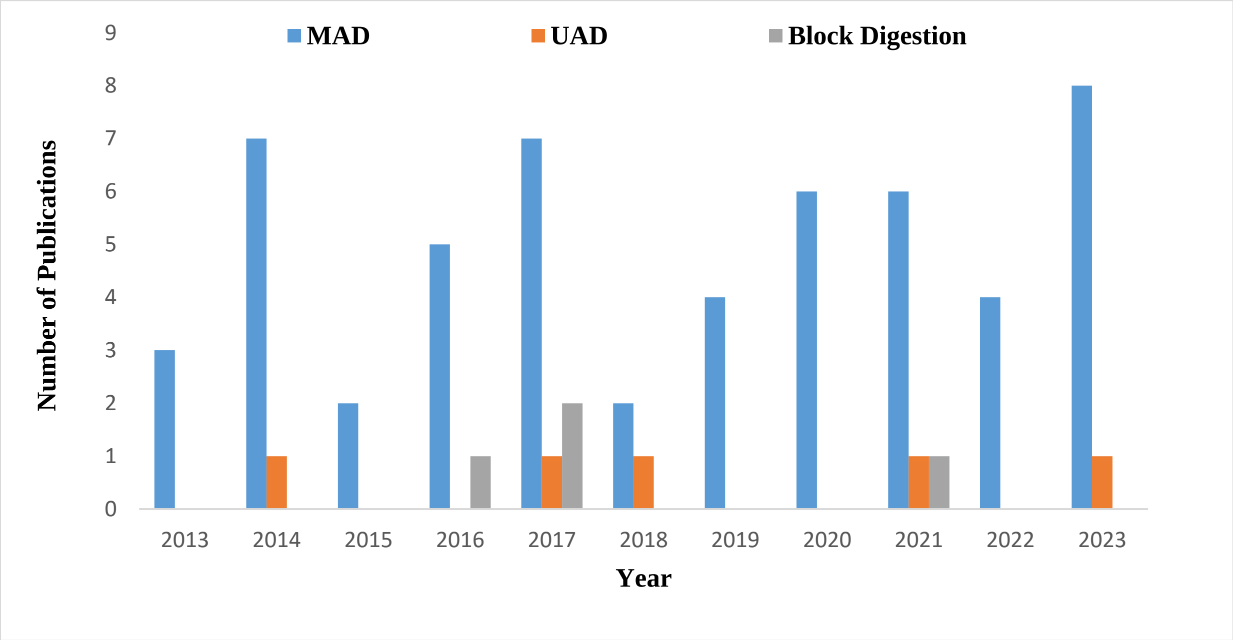

Figure 2.4: Trends in publications under the topic of wet chemistry of metals in food samples using Web of Science

Concentrated oxidizing acids are preferred digestion solvents in wet digestion methods, owing to their strong oxidizing properties. Oxidizing acids are usually used as a combination of two or more to increase the oxidation process (El Hosry *et al.*, 2023; Oreste, 2016). Commonly used oxidizing acids for wet digestion of metals are sulfuric acid (H_2SO_4), hydrogen peroxide (H_2O_2), nitric acid (HNO_3), hydrofluoric acid (HF), and perchloric acid ($HClO_4$) (Oreste, 2016).

As demonstrated in **Table 2.1**, generally HNO_3 is the universal solvent in wet digestion, it oxidizes organic matter in food samples. Its popularity in digestion of food samples is due to its ability not to interfere with most detection techniques, its affordability, and its availability in high purity (Uddin *et al.,* 2016). Unlike some oxidizing acids, by-products of sample digestion with HNO_3 do not interfere with any detection techniques used in determination of metals (Carreira-Casais, 2021; Demirhan *et., al* 2017). As shown in **Table. 2.1** in majority of the studies, HNO_3 was used in conjunction with H_2O_2 to increase its oxidizing strength to increase digestion efficiency of metals (Oreste, 2016). Moreover, another acid, which is a strong oxidizing solvent when used in conjunction with HNO_3 is HCl. However, unlike H_2O_2, HCl is not oxidizing agent when used alone. In the past decade as shown by **Fig. 2.4** there has been a lack of interest in employing HCl and H_2SO_4 as digestion solvents in food samples, this may be because these acids are known to be prone to interfering with determination of stable compounds (El Hosry *et al.,* 2023. During sample digestion of metals, the sulfates produced by H_2SO_4 may interfere with many detection techniques used for determination of metals resulting to inaccurate results (Demirhan *et., al* 2017).

However, despite this disadvantage, H_2SO_4 has been utilized successfully in determination of metals in some food samples. Lopes *et al.,* 2017 developed a block acid digestion method for the determination of Al, Cd, Cu, Fe, Mg, Mn, Mo, Na, Ni and Ti prior to microwave-induced plasma optical emission spectrometry (MIP-OES) analysis. This study digested rice husk samples using 3.6 mL of HNO_3 and 1.4 mL of H_2SO_4 at 225 °C for 205 mins in a digester block. The method was optimized using Response surface methodology. The detection limits were below 10% and recoveries ranged between 80.2 – 113.8 %. The obtained recoveries demonstrated that this method was effective for determination of the selected metals. Although this method used H_2SO_4 for digestion, no interferences were reported from possible sulfates compounds from H_2SO_4. Oreste *et al.,* 2016 did a comparative study of three acid digestion methods for the determination of Na, Ca, Cu, K, and Fe in milk powder prior to ICP-OES. In one of the acid digestion methods, the group digested milk samples in a block digester using 10 mL HNO_3/H_2SO_4 at 350 °C. The method was evaluated for accuracy using CRM (infant milk formula). The good agreement between the measured values and certified values showed that this method is suitable for routine analysis of selected metals in milk powder samples. Furthermore, HCl, is usually used in conjunction with other oxidizing acids for digestion of samples with inorganic matter. According to some literature reports combining oxidizing acids for digestion increases digestion yields (Castro-González *et al.,* 2022; Siriangkhawut, 2017; Uddin *et al.,* 2016).

One recent study by Castro-González and colleagues, (2022), evaluated the effect of using different combination of oxidizing acids for digestion of different metals. The study employed method A (10 ml 65% HNO_3), B (a combination of 5 ml 65% HNO_3 and 5 ml 30% v/v H_2O_2), and C (5 ml 30% v/v H_2O_2) for digestion for Pb, As, Cu, Cd, Cr, and zinc Zn in milk and cheese in a microwave at 200 ℃ for 15 minutes. The combination of HNO_3 and H_2O_2 in milk digestion gave higher recoveries of Zn, Cd, and Pb.

On the other hand, higher recoveries of As were obtained when using only HNO_3. In case of Cr and Cu, the recoveries for both methods did not show much difference. In case of the cheese samples, the same observations were found for digestion of As, low recoveries were obtained using a combination of HNO_3 and H_2O_2. It was concluded from this study that HNO_3 was more effective in detection of As in milk and cheese samples. On the other hand, the combination of HNO_3 and H_2O_2 was more effective in digestion of metals. Furthermore, the effect of combination of oxidizing acids was also investigated in another study by (Siriangkhawut, 2017) using univariate mode. This study employed an acid mixture (HNO_3: H_2O_2; 2:1), H_2O_2 only, and HNO_3 only in digestion of Cd and Pb in Thai rice. The highest recoveries of both metals were obtained in the HNO_3: H_2O_2 mixture compared to using other acids alone, indicating that using HNO_3 in conjunction with H_2O_2 increased the oxidation strength of the digestion solvent, thus increasing digestion of Cd and Pb in rice samples. The study also found that combination of acid mixture gave clear digests, thus indicating complete decomposition of the rice samples because of increased oxidation strength of the solvent.

Another reported study by (Uddin *et al.,* 2016) assessed the efficiency of three combination of oxidizing acids A (HNO_3–$HClO_4$ in a ratio 2:10), B (only HNO_3), and C (a mixture of HNO_3–HCl in a ratio 1:3) in digestion of Ni, Fe, As, Zn Pb, and Cd from organic matter (herbs). It was observed from statistical analysis that method A was the most efficient method in digestion of these metals, giving the highest recoveries compared to the other two methods (B and C). Therefore, results from this study proved that there is significance influence of combination of different oxidizing acids in the digestion of different metals.

As demonstrated in **Table. 2.1** it is worth to note that most reported wet digestion methods have been employing combination of oxidizing acids in a way that one acid is highly concentrated (e.g.,65 % HNO_3), which is not in line with recent developments of aligning analytical methods with green analytical chemistry. The latter aims at reducing or eliminating use of toxic solvents and toxic waste in analytical chemistry (Demirhan *et., al* 2017). Thus, to align wet digestion methods with green analytical chemistry, use of dilute oxidizing acids is slowly gaining interest in wet digestion methods.

One study by (Tarantino *et al.,* 2017) reported use of dilute HNO_3 (4.5 mol L^{-1}) in microwave digestion of Zn, Al, Ca, As, Fe, Cu, Cd, Mn, Mg and Se in rice samples. Good recoveries were obtained in the range 80–102 %. Limit of detections were in the range 0.1-0.8 2 µg g^{-1} when using ICP-OES. When employing ICP-MS for determination of metals LODs ranged from 0.002- 0.05 µg g^{-1}. Considering advantages of using dilute acids in wet digestion this method proved to beneficial in terms of safety and using greener digestion solvents. One of the most recent studies that employed a dilute oxidizing acid (0.1 mol L^{-1} HNO_3) was done in South Korea by Lee and colleagues (2023). A fast and green MAD was developed and was applied in digestion of elements Al, As, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Ni, Pb and Zn in white and brown rice, with LODs between 0.159 -9.863 µg kg^{-1} (Lee *et al.,* 2023). The method provided low residual carbon content, and residual acidity. Furthermore, the developed method was assessed for greenness using with Eco-scale tool and gave an excellent score of 85 indicating the greenness of the method. The developed method proved to be successful and that it's possible to use dilute oxidizing acids for successful determination of metals in food samples.

Ávila *et al.,* (2017) developed a block acid digestion of Al, B, Ba, Ca, Cu, Fe, K, Mg, Mn, P, Sr, and Zn using a combination of diluted HNO_3 and H_2O_2 in dog and cat food prior to ICP-MS analysis. A full 2^4 factorial design was used to optimize the significant factors. The factors found to be significant were sample mass, solvent combination, and temperature. The limit of quantifications ranged between 0.2-51 µg/g. The efficiency of the method was evaluated by analysis of certified reference materials (tomato leaves (national institute of standards and technology (NIST) 1573), peach leaves (NIST 1547), and apple leaves (NIST 1515). The method gave recoveries that ranged between 80.2 -113.8 %, and relative standard deviations that ranged between 7.3 -14.6% indicating that the method was suitable for digestion of metals in food samples. This method was not only efficient, but it also did not used harmful reagents, generate residues and there was dissolution of the samples. Barea-Sepúlveda *et al.*, (2021) used block acid digestion for determination of Cr, As, Cd, Pb, Hg, Cu, Zn and Se in Spanish and Moroccan mushroom species prior to analysis with ICP-OES, FAAS, and ultraviolet-visible (UV-Vis) spectroscopy. Mushroom samples were mixed with 2 mL nanopure water, 2 mL HCl, and 5 mL conc. HNO_3 and digested in a DigiPREP Jr block digestion system. The efficiency of the method was evaluated by analysis of CRM (Boletus Edulis) and the recoveries agreed with CRM giving recoveries that ranged between 70-130% indicating that this method can be used for determination of metals in food samples.

Another interesting trend in determination of metals in food samples using wet digestion methods is the detection techniques. As observed in **Table 2.1** inductively coupled plasma spectroscopy is shown to be the most popular analysis technique, this might be due to its capabilities to simultaneously detect multi-elements and its good detection limits, accuracy, and precision (Bulska & Wagner, 2016; Douvris *et al.*, 2023). Moreover, essential, and non-essential metals are the most reported metals, followed by heavy metals in food samples. This because there is a general rise in reported studies for the determination of metals in food samples due to the growing interests and developments in monitoring of food safety and quality. As discussed earlier, it is worth to note that even though these wet digestion methods proved to be efficient for determination of these selected metals, they use highly concentrated oxidizing acids which are associated with generation of toxic waste, toxic by-products such as NOx which are very hazardous to handle, and not in line with green analytical chemistry (GAC). Green analytical chemistry aims to reduce toxic waste by eliminating use of toxic reagents, replacing them with more environmentally friendly solvents such as dilute acids which are classified as green reagents.

Consequently, another interesting development is the use of H_2O_2 alone as a digestion reagent, a green reagent. H_2O_2 is gaining interest as an oxidizing acid in decomposition of different sample matrices, owing to its environmentally friendly digestion by-products (Mullapudi et al., 2019). Dilute hydrogen peroxide decomposes into water and oxygen at high temperatures; hence use of this green solvent does not only reduce generated waste, but it also reduces the risk associated with handling corrosive concentrated acids, toxic residues and reduces amount of acid vapours in the laboratory. For this reason, both methods developed in this study employed dilute H_2O_2 for digestion of selected metals in pet food samples.

Table 2. 1: Wet chemistry sample preparation methods applied in various food samples prior to elemental analysis.

Sample matrix	**Reagent**	**Heavy metal**	**Detection technique**	**LOD (ng/g^{-1})**	**Accuracy (%)**	**Precision (%)**	**Ref.**
Pelleted dry dog and cat food	10 ml of 65 % HNO_3	Hg, As, Pb, Cu, Fe, Zn, Se, Mn, Ag, Ba, Be, Mo, Ni, Sb, Sn, Sr, Ti, U, and V	ICP-MS	-	87 -118	**-**	**(**Macías-Montes *et al.*, 2021)
Dog and cat food	10.0 ml of conc. HNO_3	Pb, Cd, As, Hg	-	0.06 – 3.03	-	-	(Leiva, 2019)
Dog and cat food	2.0 mol L^{-1} HNO_3 and 6% m/v H_2O_2	Al, Ca, Cd, Cr, Cu, Ba, Fe, K, Mg, Mn, P, S, Sr and Zn	ICP-OES	0.03 -87	76% - 122%	<5	Da Costa *et al.*, 2013
Wheat flour	9 mL of 8 mol/L HNO_3, and 3 mL of 33% H_2O_2	Mg, K, Ca, Al, Mn, Fe, Cu, Zn, Se, Rb, V, Cd, and As	ICP-MS	0.0001-0.63	90.43-109.76	-	(Alhagri & Albeshry, 2023)

Fish muscle, pork, chicken breast and offal, nuts, beans, milk and dairy products, grain-based samples, fruits, and vegetables.	5 ml of conc. HNO_3 and 2 ml of H_2O_2 (30%, mass/volume)	Se	ETAAS	0.99	96.13-99.60	<5	(Sherovski, 2022)
Cocoa, chocolate	3 mL of 60 % (V/V) ultrapure HNO_3 and 5 mL of deionized water	Pb, Cd, As, V, Cr, Sb and Se	ICP-MS	-	90-110	0.44.89	(Dico, 2018)
Cocoa beans, cocoa powder	6 mL of HNO_3 and 2 mL of H_2O_2	As, Cd, Pb, and Sb	ICP-MS	-	89.72 - 108.75	<20	(Mohamed *et al.*, 2020)
Shrimp and crabs	4 mL HNO_3 + 4 mL H_2O_2 + 4 mL H_2O	Cu, Fe, K, Mg, Mn, and Zn	MIP-OES	-	90 -105		(Lemos *et al*., 2019)
Black Tea	5ml of 65 % HNO_3	Cd, Cu, Ni, Pb	GFAAS	0.03 - 11	92.4-117.3	-	(Shaltout, 2013)

Milk	6 mL of 0.1 mol L^{-1} HNO_3 2 mL of 30% H_2O_2	Al, As, Ba, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Sb, Se, and Zn	ICP-OES ICP-MS	0.286-82.990	-	-	(Park & Lee, 2022)
Slim tea	conc. HNO_3 and 1 ml of a 30 % (v/v) H_2O_2	Ba, Ca, Cd, Cr, Cu, Fe, Mg, Mn, Ni, P, Pb, Sr and Zn	ICP-OES	-	95.8-108	-	(Szymczycha-Madeja, 2014)
Olives	6.3 mL of HNO_3 and 0.7 mL of H_2O_2.	Pb, As, Cu, and Fe	ICP-MS	-	88-112	<10	(Llorent-Martínez, 2014)
Pepper	4.0 mL of HNO_3 65% v/v and 4.0 mL of H_2O_2 30% w/w	Ca, Fe, K, Mg, Mn, P, Zn	ICP-OES	0.7 - 4	84.61 – 118.19	-	(Gamela, 2020)
Animal feed	4 mL HNO_3 7 mol L^{-1} and 1.5 mL of concentrated H_2O_2	Ca, Co, Cu, Fe, K, Mg, Mn, Mo, Na, P, Sr, Zn	ICP-OES, ICP-MS	0.05 - 58	-	2.2 – 7.0	(Savio, 2019)
Thai rice	conc. HNO_3: H_2O_2, and H_2O	Cd, Pb	UAD	0.06 - 0.11	97.2 – 98.5	2.3 - 3.5	(Siriangkhawut, 2017)

Spirulina dietary supplements	5% v/v HNO_3	Al, Ba, Ca, Cd, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, V, Zn	UAD	0.57 - 831.74	85 - 115	1.137 – 8.72	(Neher, 2018)
Edible sea grapes	-	Cd, Pb	UAD	0.58 – 8.87	-	2.4 – 2.9	(Mayotha, 2023)
Brazilian wine	2.3 mol L^{-1} HNO_3	Al, Mn, Pb, Cu, Cd, Zn	UAD	-	-	-	(Carneiro, 2021)
Honey	6ml mixture of 4M HNO_3, 4M HCl and 0.5M H_2O_2	Zn, Fe	UAD	-	-	-	(Durrani 2014)

Notes: [FAAS]-flame atomic absorption spectrometry, **[GFAAS]-** graphite furnace atomic absorption spectrometry, **[ICP-OES]-**inductively coupled plasma optical emission, **[ICP-MS]-**inductively coupled plasma mass spectrometry, **[MIP-OES]-** microwave-induced plasma optical emission spectrometry.

2.1.2 Extraction methods

Extraction methods involve the use of extraction solvent to separate the analytes from sample matrix at medium temperatures, usually the sample matrix is not decomposed (Pico, 2013). Extraction sample preparation methods also make use of oxidizing acids to decompose organic matter in food samples. It is worth to note that extraction sample preparation methods are very similar in principle to wet digestion methods; the difference is that in acid extraction organic matter of food samples is not decomposed as compared to wet digestion where the organic matter is completely decomposed (Pico, 2013). Unlike in wet digestion where the by-products are usually clear liquids, by products of extraction methods are usually cloudy, they are not clear, showing that the organic matter of the sample was not decomposed (Pico, 2013). Temperatures employed in extraction methods are usually mild compared to wet digestion methods, this explains the complete decomposition of matter in wet digestion by-products. Like wet digestion methods, extraction methods are normally carried out in closed vessels, and they employ different types of heat such as microwave heat (microwave assisted extraction), ultrasonic heat (ultrasound assisted extraction), and pressurized hot water heat (pressurized hot water extraction) (Xia et al., 2019)

Microwave-assisted extraction (MAD)

Microwave-assisted extraction employs microwave heat energy, it is essentially the same in principle as the MAD, except that in MAE usually the sample matrix is not destroyed, and lower temperatures are usually applied compared to MAD (de la Calle, 2017; Gomez, 2020). The advantages of MAE are low cost, higher extraction yield, reduced solvent usage, and reduced extraction time (Ekezie, 2017). As observed in **Table 2.2**, MAE has been successfully used in extraction of metals in food samples; however, MAE has not been reported in literature for extraction of metals as much as MAD. Literature survey from Web of Science data base with key words, *"microwave extraction", "food analysis"* and *"metals"* covering last decade showed less publications of MAE application in analysis of metals in food compared to MAD. According to Web of Science data base, MAE is largely reported for extraction of bioactive compounds mostly in plant sample matrices.

Ultrasound assisted extraction (UAE)

Ultrasound assisted extraction is essentially the same as the UAD in principle, except that UAE does not completely dissolve the organic matter of the food sample or at least most of it in a solvent (Carreira-Casais *et al.,* 2021). Factors such as sample matrix, sample mass, ultrasonic temperature, ultrasonic power, time, solvent concentration, and solvent type may affect the efficiency of an UAE method. Proper selection of the type and concentration of solvent used being the most critical to UAE extraction efficiency (Zou *et al.,* 2013). The selection of the solvent is based on the solubility of target analyte and the interaction between solvent, and the food sample matrix. The UAE method has an advantage over MAE, because it has better protection of sample matrices because of lower temperatures generated in the medium compared to slightly higher temperatures generated in MAE medium. (Ghafoor *et al.,* 2009; Zou *et al.,* 2013). UAE has also been reported in the extraction of essential and non-essential metal elements from various food samples as shown in **Table 2.2**. According to Web of Science data base, as shown in **Fig. 2.6,** the number of publications of application of UAE in food samples has increased from 2018 to date, and this could be due to the increase in interest in GAC**.**

Pressurized hot water extraction (PHWE)

Pressurized hot water extraction is an extraction technique that utilizes pressurized liquid water as an extraction solvent above 100 °C but below 374 °C. Other common names for PHWE are subcritical water extraction (SWE), and pressurized liquid extraction (PLE) (Plaza & Turner, 2015). The PHWE was developed by Hawthorne and colleagues in mid-1990s (Hawthorne *et al.,* 1994; Yang et al., 1995). Extraction of the sample takes place in an extraction cell as shown in **Fig. 2.5**. This extraction technique has been utilized before in the extraction of various analytes in different matrices (Herrero *et al.,* 2013; Kronholm *et al.,* 2007; Saldaña & Valdivieso-Ramírez, 2015; Teo *et al.,* 2010; Wijngaard *et al.,* 2012). The advantages of this extraction technique is that it is efficient, fast, environmentally friendly, and relatively easy (Gbashia et al., 2019), however this technique has also its own limitation such as chemical reaction in sample matrix, decreased selectivity of the targeted analytes and degradation of sample analytes (Plaza & Turner, 2015).

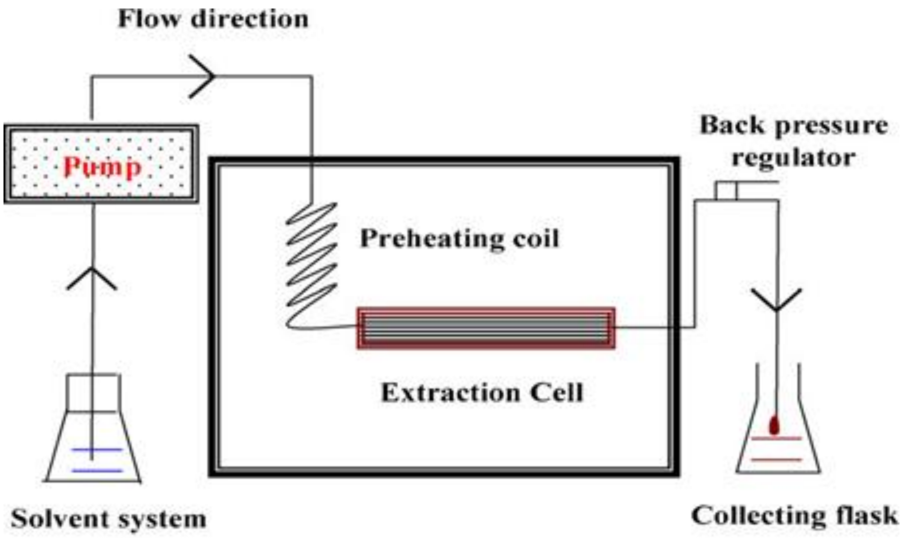

Figure 2.5: A schematic diagram of pressurized hot water extraction (PHWE) system set up.

Like wet digestion methods, extraction methods use oxidizing acids for extraction of metals, namely: HNO_3, H_2O_2, H_2SO_4, HCl, $HClO_4$ and HF. These acids maybe be used in conjunction with other acids to increase oxidation strength. As observed in **Table 2.2**, most extraction methods in food also utilize concentrated oxidizing acids**.** A study by (Blicharska, 2015) used microwave acid extraction to extract Zn, Cr, Ca, Mg, and Se from vegetable sprouts using 10 ml 65% HNO_3 prior to analysis with high-resolution continuum source atomic absorption spectrometry (HR-CS AAS). The efficiency of the method was evaluated using CRMs, and the recoveries obtained were above 98% and the RSD was less than 5%. The method also demonstrated to be efficient and moderately rapid (35 mins) in extraction of the selected metal ions, however it used highly concentrated HNO_3 acid (65%), which is associated with toxic residues. Another study by (Gu, 2015) investigated ultrasound acid assisted extraction of Cd, Cr, Cu, Ni, Mn, and Zn from fish using 8 mL HNO_3 acid (65%) and 2 mL H_2O_2 (30%) prior to AAS analysis. The recoveries obtained were good in the range of 91-109%. Detection limits in the range 0.003-0.010 mg/L and the RSDs in the range of 1.3-5.7 %, proving that this method could be used in the determination of metals in food samples, but it could also use improvements on reduction of concentration of solvents.

To align with GAC, extraction methods are slowly introducing green extraction solvents in extraction of different analytes in different samples matrix such as water, dilute HNO_3, H_2O_2, etc. Mullapudi and colleagues, (2019) developed a green, simple, and rapid microwave assisted extraction method for extraction of Zn, P, Cd, Pb, Fe, Mn, Mg, Cu, Ca, Al, Na and K from brown bread, skimmed milk powder and wheat flour, brown bread using 2 % $HClO_4$ and 4 % H_2O_2 prior to ICP-OES analysis. The digestion was successfully completed within 15 mins and recoveries for all metals were above 95 %. The short extraction time and high recoveries yields demonstrated that this method could offer very attractive benefits in determination of selected metals in similar food samples. The method did not only offer rapid digestion of samples, but it also performed very well using dilute acids. In another study by Ul-Haq and colleagues, (2021) UAE was reported for successful extraction of essential metals (Mn, Fe, Cu, and Zn) and non-essential metals (Al, Cr, As, Cd, and Pb) in vegetables (carrot, reddish, cauliflower, pumpkin, and spinach) using green solvents 5.0 mL of HNO_3 (0.5 M)-H_2O_2 (10 %) prior to FAAS analysis with recoveries of 96.0-108.3% and RSD value of ≤ 2.61, demonstrating the high efficiency of the method (Ul-Haq *et al.*, 2021). Another green extraction method (UAE) has been recently reported by (Alves *et al.*, 2023) in extraction of Mn, Mg, Fe, Ca in cane syrup prior to FAAS analysis. It is worth noting that even though this method used greener solvents (water and 1.19 mol L^{-1} HNO_3) it still reported good LODs (0.266-0.284 $\mu g\ g^{-1}$), LOQs (0.144-0.81 $\mu g\ g^{-1}$) and RSD (0.44-2.11%) indicating that this method could be suitable for extraction of the selected metals. This method did not only give high extraction efficiency, but it also used a combination of two green, environmentally friendly solvents (water and 1.19 mol L^{-1} HNO_3). As observed in **Table 2.2**, an extraction technique that is already in alignment with GAC, is PHWE, it is efficient and employs green extraction solvent (hot water). Water is not only the safest solvent but it's also the cheapest. Moreover, PHWE has been used for extraction of micro-nutrient elements (Al, Co, Cr, Ca, Fe, Ni, Zn) and macro-nutrient elements (Ca, K, and Mg) at 50–200 °C for 5–60 mins in *Moringa Oleifera* leaves (used in nutritional supplements), giving recoveries of 21-46 % and 88-98 %, respectively (Nuapia, 2020). In a recent study by (Zwane *et al.*, 2023), PHWE (50–150 °C) was successfully applied in extraction of macro-nutrients (Ca, K, and Mg) and micro-nutrients (Al, Co, Cr, Fe, Ni, Zn) from *Stevia Rebaudiana* (a herb used in sweeteners), giving recoveries of 82-91 %. The efficiency of these methods was evaluated with CRMs and the high recoveries proved that these methods could be used for extraction of metals in food with similar organic matter. Not only were these methods efficient but used hot water. It is worth noting that as shown in **Table 2.2,** PHWE was not reported in extraction of metals in food samples until recently (2020), however its slowly gaining popularity, this could be attributed to the recent growing interest in development of green sample preparation methods.

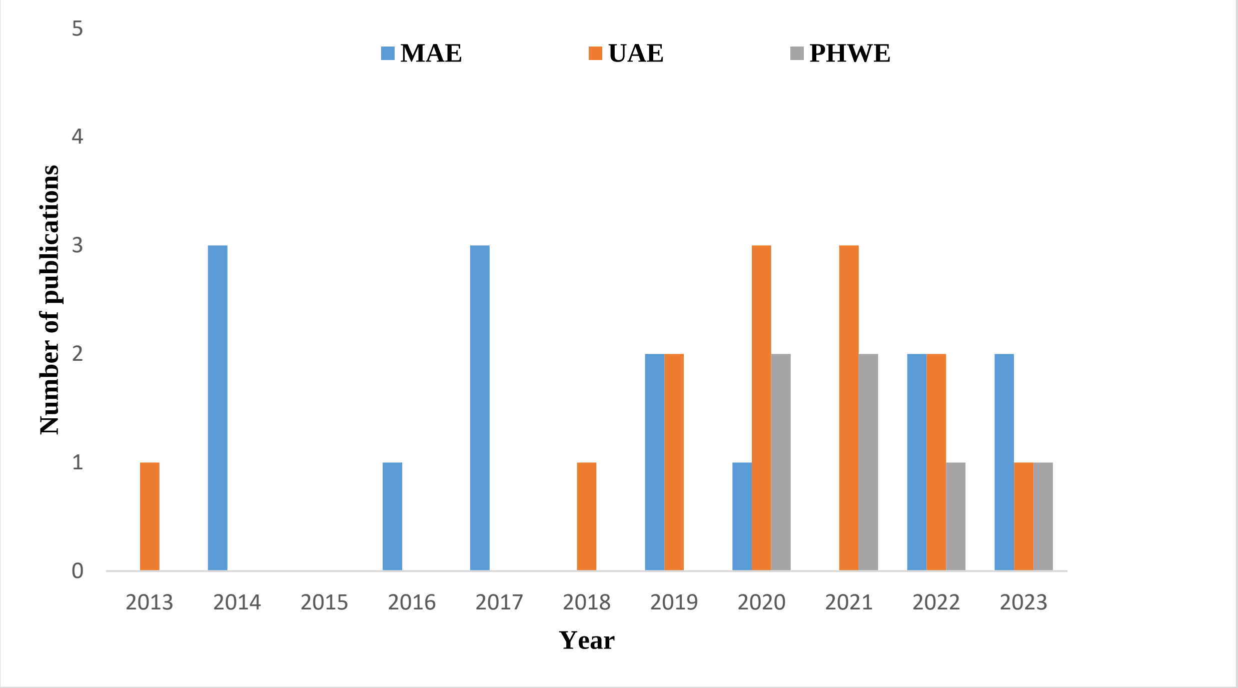

Figure 2.6: Trends in publications under the topic of extraction of metals in food samples using Web of Science.

Table 2. 2: Extraction methods applied in various food samples prior to elemental analysis.

Sample Matrix	**Solvent**	**Heavy metal**	**Detection technique**	**LOD (ng/g^{-1})**	**Accuracy (%)**	**Precision (%)**	**Ref.**
Brown bread, skimmed milk powder and wheat flour, brown bread	2% $HClO_4$ + 4% H_2O_2	Zn, P, Cd, Pb, Fe, Mn, Mg, Cu, Ca, Al, Na and K	ICP-OES	-	> 95	-	(Mullapudi *et al.*, 2019)
Cassava peel samples used in animal food	6.3 mL HNO_3, 2.1 mL HCl, and 1.7 mL CH_3COOH	Ca, Mg, Cu, Mn, Fe, and Zn	FAAS	0.4 -10.4	96.8 – 101.2	1.9-3.8	(Costa *et al.*, 2018)
Meat	10 mL of HNO_3 1 mol L^{-1}	As	HG-AFS	0.013		0.3	(Ruiz-de-Cenzano, 2017)
Vegetable sprouts	10ml 65% HNO_3	Zn^{2+}, $Cr^{3+,}$ $Ca^{2+,}$ Mg^{2+}, and Se^{2}	HR-CS AAS	-	> 95	< 5	(Blicharska, 2015)

Milk and fermented milk products	3 ml of diluted HNO_3	Cd, Pb, Cu	Potentiometric Stripping Analysis (PSA)	0.30 - 3.8	97.1 – 98.7	5.8-8.7	(Suturović, 2014)
Cane syrup	5.0 mL solvent: Ultrapure water for (Fe and Mn) HNO_3:H_2O (75:25%, v/v) (Mg and Ca)	Mn, Mg, Fe, Ca	FAAS	0.266-0.284	-	0.44-2.11	(Alves *et al.*, 2023)
Carrot, reddish, cauliflower, pumpkin, and spinach	5.0 mL of HNO_3 (0.5 M) - H_2O_2 (10 %)	Mn, Fe, Cu, and Zn, Al, Cr, As, Cd	FAAS, ICP-OES	-	96 – 108.3	≤ 2.61	(Ul-Haq *et al.*, 2021)
Pepper	14 mL of HNO_3 1 mol L^{-1}	Ca, Fe, K, Mg, Mn, P, Zn	ICP-OES	0.5 - 4	84.61 – 118.19	-	(Gamela, 2020)
Sugar	60:40% (v/v) of HNO_3:H_2O_2	Mn, Zn, Fe, Mg, Ca	FAAS	0.0078 – 0.1168	91–109	0.63 – 5.74	(Dos Santos, 2019)

Soybean, canola, and sunflower oils	0.5 mol L $^{-1}$ HCl	Cu, Fe, Ni, Zn	HR-CS FAAS	1.4 – 3.6		0.04 - 0.21	(Trindade, 2015)
Fish	5 mL of HNO_3-H_2O_2	Cd, Cr, Cu, Ni, Zn	FAAS	0.003-0.010	91 - 110	1.3 -5.7	(Gu, 2013)
Bee Pollen	1.1 mol L^{-1} HNO_3 (Ca, Cu, Mg, Mn, and Zn) 0.9 mol L^{-1} HNO_3 (Fe)	Ca, Cu, Fe, Mg, Mn, Zn	FAAS	-	-	2 - 4	(Pohl, 2020)
Guarana fruit	3.1 mL of 4.5 mol L^{-1} HNO_3 and 6.9 mL of ultrapure water	Ca, Mg, K, P, S, Fe, Cu, Mn	ICP-OES	0.03 - 1.1		< 8	(Farias, 2022)
Pepper	Acetic acid solution	K, Na, Ca, Mg, Zn, Cu, Fe, Mn, Cr, Se, Mo, Li, Al, B, Pb, Hg, Cd, As, Ni		-	-	-	(Lučić, 2022)

Soya bean seeds	10.0 mL of 3.5 mol L^{-1} HNO_3	Cd, Cu, Ni, Pb, Zn	FAAS, EAAS	0.002 – 0.160	-	>8	(Machado, 2019)
Infant milk	2 mL HNO3 and 1 mL H2O2.	Ca, Cu, Fe, K, Mg, Mn, Na, P and Zn	ICP-OES	0.08 – 0.1	74 - 102	-	(Fioravanti, 2020)
Stevia Rebaudiana (a herb used in sweeteners	Hot water	Ca, K, and Mg, Al, Co, Cr, Fe, Ni, Zn	ICP-OES	-	82 - 91	-	(Zwane, 2023)
Moringa Oleifera leaves (used in nutritional supplements)	Hot water	Ca, K and Mg, Al, Co, Cr, Cu, Fe, Ni and Zn	ICP-OES	-	21 - 98	-	(Nuapia, 2020)
Edible seaweed	Hot water	As, Ba, Cd, Cr, Mn, Ni, Pb, Zn	ICP-MS	0.02 – 2.2	93 - 117	-	(Rey-Rubio, 2012)

Edible brown seaweed	Hot water	As, Cd, Hg, Pb, Ca, Mg, K, Na, Cu, Fe, I, Mn, Zn, Al	ICP-MS	-	-	-	(Saravana, 2016)

Notes: [FAAS]-flame atomic absorption spectrometry, **[GFAAS]-** graphite furnace atomic absorption spectrometry, **[ICP-OES]-**inductively coupled plasma optical emission, **[ICP-MS]-**inductively coupled plasma mass spectrometry**, [PSA]-**Potentiometric Stripping Analysis, **[HR-CS AAS]** High-resolution continuum source AAS, **[FAAS]-** flame atomic absorption spectroscopy, **[HG-AFS]-** Hydride generation atomic fluorescence spectrometry.

2.1.3 Combustion methods

The combustion methods such as microwave induced combustion (Crizel, 2015; Mesko, 2010; Muller, 2013; Pereira, 2013; Picoloto, 2016) and oxygen flask combustion involve complete decomposition of the sample matrix in a vessel in the presence of oxygen at extremely high temperature (~ 500 °C)(Zhou, 2016). Combustion of food samples may be done in closed or open vessels. Once combustion is completed, the products of combustion are dissolved in a solvent. The isolated analytes of interests are usually present as gaseous, non-volatile, and volatile compounds (Crizel, 2015). Like wet digestion and extraction methods, combustion uses acids for dissolving by-products of combustion such as HNO_3, HCL, HF, H_2SO_4 etc.

Microwave induction combustion (MIC)

Microwave induction combustion is one of the simplest preparation methods which involve decomposition of organic material by reacting with oxygen at relatively high temperatures in a closed quartz pressurized vessel (Barin, 2014). Microwave induction combustion is widely reported in literature for sample preparation methods for organic material including food for further halogen determination. Microwave induction combustion has been used as a sample preparation methods of food samples such as bovine liver, corn starch, milk powder, and wheat flour (Mesko, 2010), pet food (Crizel, 2015), and sea food (Picoloto, 2016). One of the biggest advantages of MIC is its faster reaction rates compared with the other sample preparation methods. The limitations of MIC are that its expensive equipment and it's not feasible for reaction monitoring (Barin, 2014).

Oxygen flask combustion

Oxygen flask combustion also known as a Schoniger Oxygen Flask was invented by Wolfgnag Schoeniger in 1955. It is a well-documented combustion technique for combusting food samples and other sample matrices prior to elemental analysis, especially analysis of the halogens. The principle of oxygen flask is very simple, as shown in **Fig. 2.7** it involves combustion of the sample matrix in a heavy walled 500 mL conical flask filled with oxygen with a hard glass stopper. A platinum holder is attached to this stopper and contains an ashless paper holder which in turn contains a weighed-out sample to be combusted. A few millilitres of absorbent liquid are placed in the flask. Once the sample is combusted, the combusted products are absorbed into the absorbent liquid. The attractive features of oxygen combustion method are its ability to cope with a wide range of sample matrices and its cheap set up costs compared to sample preparation methods which used expensive instrument such a microwave, ultrasound etc.

Over the past decade, performance of combustion in open vessels has greatly decreased, this is due to the risks associated with combustion of samples in open vessels (Pereira, 2013). Open vessels combustion is prone to contamination and loss of volatile analytes. Nevertheless, as shown **in Fig. 2.8**, combustion of food samples in closed vessels such as MIC and oxygen flask combustion is still being reported although it’s not widely reported compared to wet digestion methods (Pereira, 2013). The reason may be due to recent developments and improvements in wet digestion and extraction methods which offer attractive benefits when compared to other sample preparation methods. However, MIC and oxygen flask combustion is still being applied in digestion of samples that are hard to digest using wet digestion and extraction methods, such as fuels, and fluoropolymers. Some studies have been reported comparing the efficiency of combustion methods with wet digestion and extraction methods.

A study by Maciel and colleagues *(*2014) compared performance of microwave induced combustion and microwave assisted digestion for digestion of Cd, Co, As, Se, Mo, Zn, Fe, Cu, Mn, and Cr in fish samples prior to ICP-OES. In MIC, 5 mol L^{-1} of HNO_3 at 1400 °C was used as an absorbing solution and 6 mL of 14 mol L^{-1} HNO_3 at 200 °C as a digesting solvent in MAD. It was observed that both sample preparation methods were suitable for the digestion of the selected metals in fish meal, however MIC gave better recoveries and limits of detection compared to MAD. The reason for better performance of MIC may be attributed to the high temperatures of combustion (up to 1400 °C) which were employed in MIC which ensured complete oxidation of the organic matrix of fish samples. It was reported that after MIC, the resulting absorbing solution was clear, indicating complete oxidation of the sample organic matter. Furthermore, it was reported that the extreme temperatures did not damages the vessels. Although MAD employed moderate temperatures, it used a highly concentrated solvent which is not favoured in the recent developments of sample preparation methods (Muller *et al.,* 2013). It may also be concluded from this study that the use of highly concentrated digestion solvent does not necessarily result in good recoveries, temperature also plays an important role. Hence there should be balance of the two, together with other factors to achieve good recoveries. Another study by Crizel and colleagues (2015), compared the efficiency of MIC method with a method recommended by Association of Official Analytical Chemists (AOAC) for determination of Cl in pet food samples. The obtained recoveries for both methods agreed (> 97 %). The agreement of recoveries of both methods and the high recoveries obtained it can be concluded that this method is suitable for determination of Cl in pet food.

Other researchers have developed combustion methods and used CRMs for evaluating method efficiency without comparing it with other methods. For instance, (Zhou, 2016) developed modified oxygen combustion method for combustion of tea samples for determination of 15 rare earth elements using 5 mL of 4% HNO_3+1% HF (v/v) as an extractant prior to ICP-MS analysis. CRMs were used for evaluating the efficiency of this method and measured results agreed with CRM results, giving recoveries above 90%, RSDs ranging between 2.7 -5.5%, and detection limits ranging between 0. 001 mg/kg - 0. 006 mg/kg. The obtained recoveries were good, demonstrating the suitability of this method for the combustion of food samples. Another study by Pereira and colleagues (2013), developed modified microwave induced combustion for determination of Ba, Ca, Co, Cr, Cu, Fe, Mg, Mn, Sr, V and Zn in milk powder prior to ICP-OES analysis. The milk samples were combusted in a glass vessel under oxygen flow rate to 15 l min^{-1} using 50 µl of 6 mol L^{-1} ammonium nitrate as an ignition aid. Obtained recoveries were above 95 % demonstrating high efficiency of the method. In another study (Muller *et al.,*2013). MIC was used for sample preparation in determination of As, Cd, and Pb in nuts prior to analysis with ICP-OES. The LODs obtained were 3, 2, and 6 $ng.g^{-1}$ for As, Cd, and Pb, respectively. The high recoveries of this method also demonstrated the suitability of this method for application in determination of metals in food samples.

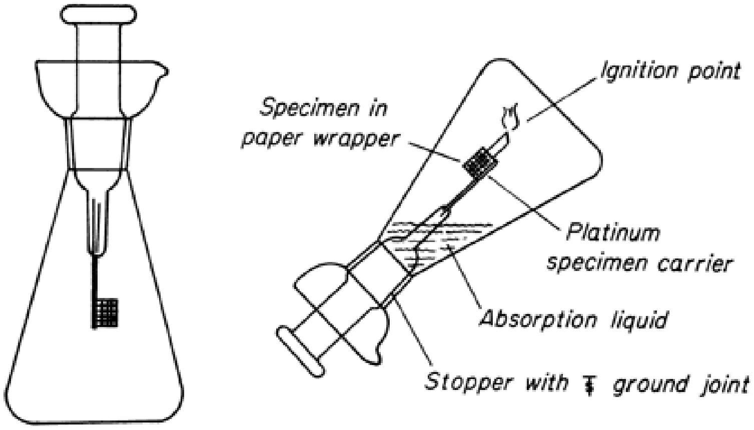

Figure 2.7: A schematic diagram of oxygen flask combustion (Kumari et al., 2022)

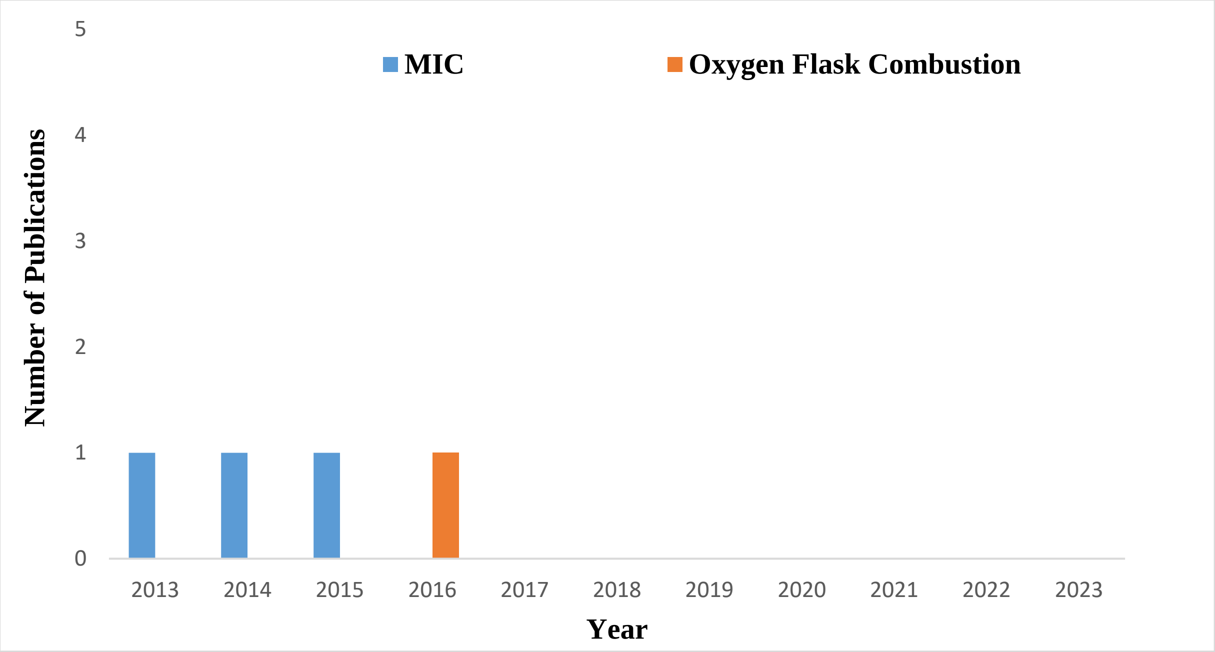

Figure 2.8: Trends in publications under the topic of combustion of metals in food samples using Web of Science.

Even though the above-mentioned sample preparation methods have several reported limitations as discussed above, the limitation which has gained a lot of interest in the past decade is the use of highly concentrated acids as solvents for extraction and digestion by most of these sample preparation methods (Bizzi et al., 2017). Concentrated acids such as nitric acid produce carcinogenic nitrous oxide (NO_x) to the atmosphere during digestion and extraction process (Bizzi et al., 2017). To solve the challenges of concentrated acids in extraction and digestion methods, use of greener extraction and digestion solvents such as dilute hydrogen peroxide have been recently introduced. The advantage of using greener H_2O_2 as a solvent is that it is converted to environmentally friendly components during extraction and digestion (Demirhan et., al 2017). Dilute hydrogen peroxide decomposes into environmentally friendly water and oxygen at high temperatures; hence use of this solvent does not only reduce generated toxic waste and lower solvent use, but it also reduces the risk associated with handling corrosive concentrated acids, toxic residues and reduces amount of acid vapors in the laboratory (Demirhan et., al 2017). Therefore, this study chose the use of dilute hydrogen peroxide (5M) as a digestion and extraction solvent in microwave digestion and ultrasound extraction of heavy metals in pet food prior to ICP-OES analysis. The microwave and ultrasound were selected instruments owing to their modern technology and attractive benefits compared to other techniques such as their rapid capabilities in digestion and extraction of metal analytes. Additionally, digestion in enclosed microwave PTFE vessels does not only minimize contamination of the sample from the atmosphere, but it also minimizes loss of some sample components which can evaporate during digestion in an open digestion vessel. Both MAD and UAE are timesaving. The microwave (Anton Paar, Multiwave 5000) utilized in this study can simultaneously digest up to 24 samples and reaction times can be as little as 10 minutes compared to more than 1 hour or more for traditional solvent digestion techniques. ICP-OES was selected as the analytical tool due to its fast multi-elemental analysis capabilities, and easily adjustable wavelength lines to obtain required sensitivity.

Therefore, considering the above review of development, and application of these sample preparation techniques, together with their limits and demerits, it can be concluded that wet digestion methods, followed by extraction methods have been and are currently (2013-2023) most widely applied techniques. It was also observed from reported literature that a general trend in both wet digestion and extraction methods is the new developments of attempting to align these methods with GAC, mostly by using greener solvents. Hence this trend of development of greener sample preparation methods using green digestion and extraction solvents has led to an increase in development of metric tools to assess the greenness of methods.

2.2 ASSESSMENT OF GREENNESS OF METHODS

Greenness metric tools are used for assessment of greenness of methods, thereby evaluating the impact of an analytical method on the environment, human safety, and human health (Kowtharapu, 2023). Greenness metric tools have been widely reported on assessment of analytical methods used for determination of organic compounds, especially, in pharmaceutical applications. However, greenness metric tools have not been reported on analytical methods used for determination of metals in food samples. One study reported the determination of sulfadiazine and trimethoprim in bovine meat and chicken muscle (Mohamed, 2020), 13 biogenic amines in homemade wine (Płotka-Wasylka, 2016), 9 sulfonamides in milk and beef (Duan, 2020), fluoroquinolones in chicken livers (Moema, 2023), antibiotic residues in food of animal origin (eggs, milk, liver, kidneys) (Vakh, 2023), and steroids water samples (El-Deen, 2019). There is little reported information on assessment of greenness of analytical methods for determination of metal ions in food samples. The most used greenness metric tools that have been reported in literature, include Eco-Scale (ESA), Green Analytical Procedure Index (GAPI), NEM and AGREE (Kowtharapu, 2023), and AGREEprep (Wojnowski *et al*., 2022).

2.2.1 National Environmental Methods Index (NEMI)

National Environmental Methods Index (NEMI) is widely reported in literature for assessment of analytical methods used in determination of organic compounds (Moema, 2023; Mohamed, 2020). NEMI is described in a pictogram that has 4 parts, that relate to different acceptance criteria (Stojanović, 2023). NEMI metric tool has been used in assessment of analytical methods such as hollow fiber liquid phase microextraction coupled with HPLC (Moema, 2023), Micellar Liquid Chromatography (MLC) and Ultra performance Liquid Chromatography-Mass Spectroscopy (UPLC-MS) (Mohamed, 2020). NEMI is one of the most simple and easy to use tools, however it’s biggest limitation is that its results of analysis are qualitative only (Stojanović, 2023). This tool is based on the reagents used and it does not consider other important aspects of an analytical methods such as sample collection, energy consumption, waste, transportation (Stojanović, 2023).

2.2.2 Eco-Scale Assessment (ESA)

Eco-Scale Assessment (ESA) is a semi-quantitative green metric tool which was initially proposed in 2012 (Gałuszka, 2012) for assessing greenness of analytical methods. It considers more environmental parameters than NEMI. ESA is built on assigning penalty points subtracted from a base of 100 for parameters of analytical technique that are not in agreement with green chemistry (Kowtharapu, 2023). Therefore, the higher the score, close to 100, the greener and more economical is the analytical method. Eco-scale is one of well documented greenness assessment tools. It is well reported in assessment of newly developed chromatographic methods, especially, in pharmaceutical applications (Hafez, 2023). It has been reported in the assessment of greenness of developed RP-HPLC method (Mohamed, 2016), micellar Organic-solvent free HPLC method (Hafez, 2023), green HPLC method (Duan, 2020) and eco-friendly HPLC method (Elmansi, 2019). ESA is considered comprehensive, easy and quick to use, however its biggest limitation is that it is generic, it does not provide qualitative information of an analytical method (Stojanović, 2023).

2.2.3 Green Analytical Procedure Index (GAPI)

Green Analytical Procedure Index (GAPI) is one of the recently proposed greenness assessment tools. It was proposed in 2018 by (Płotka-Wasylka, 2018a), it assesses the green character of the an analytical method, from sample collection to sample analysis (Płotka-Wasylka, 2018b). GAPI was created using other assessment tools such as the ESA, to provide not only basic information but also qualitative information of an analytical method (Stojanović, 2023). GAPI is well documented in literature for assessment of analytical methods for determination of organic compounds, largely in pharmaceutical application. GAPI has been reported in assessment of greenness of an environmentally friendly HPLC (Saraya, 2023), dispersive liquid-liquid microextraction based on solidification of floating organic droplet (DLLME-SFOD) (El-Deen, 2019). It has also been reported in extraction methods (UAE and MAE) using natural deep eutectic solvents (NADES) (Ferreira, 2022). The biggest merit of this tool is its completeness, it assesses all parts of an analytical procedure (Stojanović, 2023). Other advantages of this tool is it's visual presentation of the performance of an analytical method, which allows for an at-a-glance of comparison of different analytical methods and it clearly identifies the weakest points of an analytical method (Płotka-Wasylka, 2018b).

2.2.4 Analytical GREEnness Metric Approach (AGREE)

The metric system of an assessment of an analytical methods' greenness must be characterized by flexibility of input importance, simplicity of output, clarity of output, and comprehensiveness of input data (Wojnowski *et al*., 2022). To meet these requirements, in 2020, (Pena-Pereira, 2020) proposed a novel metric tool for assessment of greenness of analytical methods (AGREE). AGREE is anchored on 12 principles of Green analytic chemistry, it converts the 12 principles of GAC into scores, which range between 0-1. Each score contributes to the overall score of the analytical methods. The assessment output is depicted by a clock-like graph, with the overall score of assessment and color in the middle. The first application of AGREE was in assessment of greenness of three extraction methods, used for determination of polybrominated diphenyl ethers (PBDEs) in soil, with final assessment scores of 0.43, 0.37, and 0.23 (Pena-Pereira, 2020). Recently AGREE was used for assessment of greenness of TLC and HPLC–PDA chromatographic techniques developed for simultaneous analysis of levamisole with triclabendazole in pharmaceuticals with final assessment scores of 0.8 and 0.71, respectively (Attia, 2023). In another study by Habeeb and colleagues (2023), AGREE was successfully applied in assessment of greenness of an electro-driven separation method for simultaneous analysis of 2 antibiotics and obtained a perfect greenness of 0.94. AGREE is comprehensive as it incorporates all of 12 principles of GAC, it's easy to use (simple software), and it's easy to interpret its results depicted by a colored pictogram, showing the weak and strong points of an analytical method.

2.2.5 AGREEPrep- Analytical Greenness Metric for Sample Preparation

In the past decade the above-mentioned greenness assessment metric tools have been created to assess and the compliance of analytical procedures with GAC principles, however similar metric tools for assessing the greenness of a sample preparation step have not been reported in literature (Wojnowski *et al.*, 2022). To bridge this gap in the previously created metric tools, in 2022 Wojonowski and colleagues developed a user-friendly tool to assess greenness of sample preparation methods. Thus AGREEPrep is a metric tool (software) to assess the greenness of the sample preparation stage of an analytical procedure (Wojnowski *et al*., 2022). It uses 10 individual (criteria) steps for assessment and each step requests input of data (Wojnowski et al., 2022). The scores from each step are weighted and combined to give an overall score that ranges from 0 to 1. The extremes represent the worst and ultimate performance of a method, respectively. After completion of assessment, the metric tool generates a round pictogram with a circle that's shows the overall score of the method and ten trapezoid bars corresponding to the ten steps(criteria), each having a length equivalent to the assigned weight. The color in each criterion changes after evaluation, thus providing a way to identify the strong and weak points of a procedure, and their contribution to the overall score (Wojnowski et al., 2022). In this present work, AGREEPrep was used in assessment of both developed sample preparation methods.

Apart from the greenness, it is crucial to also investigate the most influential parameters for a newly developed method, so that it can perform at the optimal level (Costa et al., 2020; Lee *et al.*, 2022). Therefore, the next section describes tools used to optimise newly developed methods.

2.3 OPTIMIZATION OF ANALYTICAL METHODS IN FOOD SAMPLES

Optimization process is done to improve the performance of a process or system to yield the best response or responses of a process (Bezerra, 2019; Ferreira, 2018). It visualizes how a process's response changes with increasing or decreasing the level of its factors. When developing analytical methods, several factors may affect the performance of the method, as a result affecting the response of the method (Ferreira, 2018).. Therefore, optimization is an essential step in the development of an analytical method. There are two well documented types of optimizations, namely univariate and multivariate optimization, the latter being the widely reported optimization technique in literature (Bezerra, 2019; Costa *et al.*, 2020; Farias, 2022; Gamela, 2020; Lee *et al.*, 2022).

2.3.1 Univariate optimization

Univariate optimization, also known as one-variable-at-a-time technique is applied in systems where no interdependencies exist among factors, thereby evaluating one factor at a time, whilst all other factors are kept constant (Ávila et al., 2017; Ferreira, 2018). Thus, it solely focuses on one factor at a time, therefore it is not suitable for complex systems where interdependencies exist between different factors. The advantages of univariate optimization over multivariate optimization are its simplicity and ease of results interpretation (Ferreira, 2018). It’s easy to use since only one factor needs to be considered and results are also easy to interpret since they clearly highlight how changing one factor affects the response (Ávila et al., 2017). There are no complications resulting from interpreting the simultaneous interaction of several factors and their effect on the response. The biggest limitation of univariate optimization is that it is time consuming and requires large quantities of reagents, thereby increasing experimental costs (Barros *et al.*, 2013; Ferreira, 2018). In a world of continuously improving analytical procedures to promote green chemistry, use of large reagents is a major limitation. As shown in **Fig. 2.9,** to overcome these limitations, many researchers have now resorted to multivariate optimization.

2.3.2 Multivariate optimization**.**

Multivariate optimization, also known as multidimensional optimization, is applied where several combinations of factors affect the response of a method, it evaluates more than one factor at a time (Ferreira, 2018). The aim of multivariate optimization is to find the best optimal combination factors that will yield the best response of a method. The multivariate optimization designs can be grouped into first order and second order designs. As shown in **Fig. 2.9**, multivariate optimization has gained a lot of interest in the past decade for optimizing analytical methods.

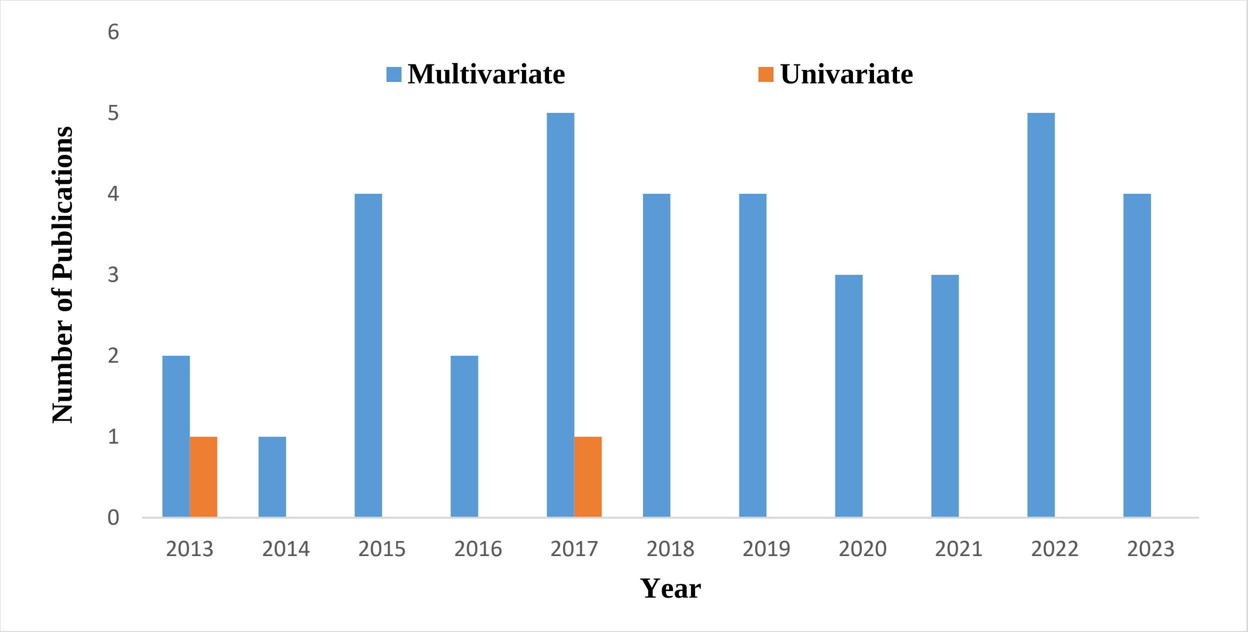

Figure 2.9: Trends in publications under the topic of optimization sample preparation methods of food samples for determination of metals.

2.3.2.1 First order experimental designs

The first order experimental designs, also known as preliminary screening designs, are used at the initial stages of an experimental design. Their aim is to narrow down the list of potentially significant factors and their combinations (Ferreira, 2018). Classical first order designs include 2^k full factorials, placket-Burman, cotter, and mixed level designs. In this present study a 2^k full factorials design was initially used to screen the potentially significant factor. A 2^k full factorials design is a *k*-factor design, where each factor has two levels, coded −1 (low value) and +1 (high value) and 2^k experimental runs are based on the 2^k combinations of the ±1 factor levels. It requires relatively few experimental runs, as it allows evaluation of multiple factors and their combination at a time, thus allowing evaluation of effects of independent factors on a dependent factor in a single experimental run (Ferreira, 2018).. This can save time, and reagents, thereby reducing costs. A major limitation of this experimental design is that it can be complex and tiring when applied in an experimental design with too many factors and levels, as the number of experimental runs exponentially increase with increase in factors and levels. Literature reports have demonstrated that 2^k full factorials are often used in preliminary screening of potentially significant factors (Chen *et al.*, 2015; Gamela, 2020; Pohl, 2020; Szymczycha-Madeja *et al*., 2015).

2.3.2.2 Second Order experimental designs

Second order experimental designs are used at later stages of experimental design when significant factors of an experimental design have been identified. Some well documented classical second order experiment designs include central composite design (CCD), three-factor factorial design, box Behnken, and Boehlert (Ferreira, 2018), just to name a few. These experimental designs are used in response surface methodology (RSM), a collection of tools that consists of mathematical and statistical techniques that help to establish linear, interaction, and quadratic effects of variables on a specific response, thereby helping to determine the optimum conditions of an analytical method (Gamela, 2020). They are used to re-study factors that were identified as statically significant in two-level factorial design. Furthermore, they establish a quadratic equation that shows the significance of these factors and their effects on the response. The quadratic equation helps to establish the maximum and minimum values of factors. Therefore, in this present study, CCD was used for further optimization of the significant factors identified in first order optimization. A CCD, also known as a Box-Wilson central composite design consists of factorial points, axial points, and central points. A CCD has been reported in literature for multivariate optimization of analytical methods for determination of various metal ions in food samples such as rice-cowpeas (Danbaba, 2015), fish (Bozorgzadeh, 2021), milk powder (Evgenakis, 2018), edible oils (Tokay, 2016), honey (Daşbaşı, 2016), and beverages (Biata, 2017). There is no reports in literature on application of CCD in pet food samples, however other types of designs such as three-level full factorial design (da Costa, 2013) have been used in multivariate optimization in pet food samples. In conclusion multivariate optimization has been and is currently being used for optimization of sample preparation methods for determination of metals in food samples.

Post sample preparation step, the commonly used analytical techniques for detection and quantification of heavy metals in food samples are flame atomic absorption spectrometry (FAAS), graphite furnace atomic absorption spectrometry (GFAAS), inductively coupled plasma optical emission (ICP-OES), inductively coupled plasma mass spectrometry (ICP-MS), and X-ray fluorescence spectrometry (XRF). Thus, the next section outlines these techniques.

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CHAPTER THREE (INSTRUMENTATION)

PREAMBLE

Chapter three provides the principles of instruments used in detection and quantification of heavy metals in food samples. It gives an overview of principle of spectroscopic techniques, flame atomic absorption spectrometry (FAAS), graphite furnace atomic absorption spectrometry (GFAAS), inductively coupled plasma optical emission (ICP-OES), inductively coupled plasma mass spectrometry (ICP-MS), and X-ray fluorescence spectrometry (XRF). It goes on to further outline the limitations and merits of these instruments.

3.1 INSTRUMENTATION OF DETECTION TECHNIQUES IN FOOD SAMPLES

The techniques for detection and quantification of heavy metals in food and other sample matrices are well documented in literature. The most common used analytical techniques for detection and quantification of heavy metals in food samples are flame atomic absorption spectrometry (FAAS) (Acar *et al.*, 2016; Da-Col *et al.*, 2009; Malhat, 2011; Nunes *et al.*, 2011; Raposo *et al.*, 2008), graphite furnace atomic absorption spectrometry GFAAS (Dos Santos, 2018; Fairulnizal, 2019), inductively coupled plasma-mass spectrometry (ICP-MS) (Alhagri & Albeshry, 2023; Gianluigi Maria Lo Dico, 2018; Lee *et al.*, 2022; Šelih *et al.*, 2014), inductively coupled plasma-optical emission spectrometry (ICP-OES) (Lee et al., 2022; Šelih *et al.*, 2014), and x-ray fluorescence spectrometry XRF (Byers, 2019; Frydrych, 2023; Gazulla, 2021; Panebianco, 2022; Perring, 2017).

Several researchers have applied these techniques, after application of various sample preparation techniques. The selection of the detection and quantification technique largely relies on the sample status and type, as some samples matrices are complicated and can cause sample matrix interferences during analysis (Agatemor & Beauchemin, 2011). However, there are other factors that are considered when selecting the technique such as the instrument sensitivity, ease of operation, speed of analysis, and cost, just to name a few (Bulska & Wagner, 2016). The proposed study aimed at using ICP-OES to analyse Cd, Pb, As, Sn, and Cr after ultrasound assisted hydrogen peroxide extraction (UA-HPE) and microwave assisted hydrogen peroxide digestion (MW-HPD). To the best of our knowledge ICP-OES has never been applied with UA-HPE and MW-HPE in determination of metals in pet food samples.

3.1.1 Flame Atomic Absorption Spectrometry (FAAS)

FAAS is one of the two types of atomic absorption spectroscopy (AAS) techniques commonly used in detection and quantification of elements in food samples. It was developed in the 1950s by Alan Walsh, however, it was commercially released as an analytical technique in 1960s (Shukor *et al*., 2015)**.** It consists of four main components: the light source, atomization system, monochromator, and the detection system (Gonzálvez & De La Guardia, 2013)**.** The basic principle of FAAS is that atoms of an analyte absorb light of specific wavelength, and when the atoms absorb that specific light, the electrons in the atom are moved from ground state to excited state. The amount of light absorbed by the analyte atoms is directly proportional to the concentration of the absorbing atoms (Fairulnizal, 2019). As shown in **Fig.3.1** during typical elemental analysis, a sample solution with an element is passed through the flame, to atomize the target analyte atom. Simultaneously, the light from a hallow cathode lamp passes through the flame, which excites the electrons, and they absorb light at specific wavelength (nm). A detector measures the analyte atoms absorbance and converts it to absorption data. To reduce background interference, a monochromator is placed between the sample and the detector. The FAAS technique is one the easily accessible techniques in most laboratories because of its low operation costs and good analytical performance, with good detection limits typically ranging from 1–100 $\mu g\ L^{-1}$. It is highly selective and sensitive and can determine a wide range of elements at trace and ultra-trace levels (Gonzálvez & De La Guardia, 2013). The biggest limitations of this technique are mono-elemental detection and limited linearity (Gonzálvez & De La Guardia, 2013). FAAS has been successfully used for detection and quantification in several food samples such as pork meat products (Barone *et al., 2021)*, tahini, wheat flour, corn flour, and rice flour (Acar *et al*., 2016), lamb meat, organic pear, radish leaf, pomegranate flower (Daşbaşı et al., 2015). There are few reports in literature for quantification and determination of heavy metals in pet food using FAAS, one study (Pereira *et al.*, 2018) reported successful mineral (sodium, calcium, iron, magnesium, and potassium) profiling of dry pet food using FAAS (Pereira *et al.*, 2018).

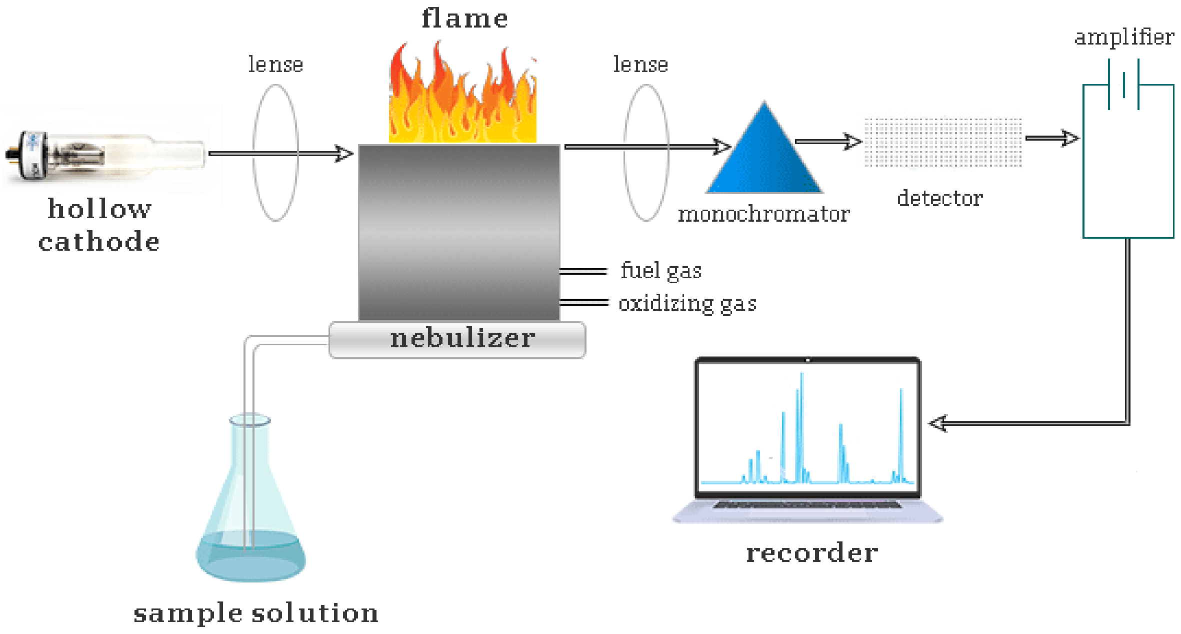

Figure 3.1: A schematic diagram of flame atomic absorption spectrometer (Adapted from (Fairulnizal, 2019))

3.1.2 Graphite furnace atomic absorption spectrometry (GFAAS)

Graphite furnace atomic absorption spectrometry is another type of AAS, also known as electrothermal atomic absorption spectrometry. It is a technique also used for detection and quantification of ultra and trace elements (de Oliveira, 2017; Fairulnizal, 2019). GFAAS is essentially the same as FAAS in principle except that GFAAS uses a graphite coated furnace to vaporize the sample instead of the flame used in FAAS (Mollo, 2017**).** Hence, it’s a non-flame AAS technique, instead of using the high temperatures of the flame for atomization of the sample. It uses electrically heated graphite tubes as shown in **Fig.3.2.** The graphite furnace temperature can reach up to 3000 °C (El Hosry, 2023; Fairulnizal, 2019). The most attractive features of the

GFAAS technique are its ability to directly analyses solid samples, highly sensitive, and small sample volume. GFAAS is 100-1000 times more sensitive than FAAS (Fairulnizal, 2019). The biggest limitation of this technique is that it's known to be prone to background and matrix effects interferences (Hill, 2016). Background effects arise from vaporized matrix components which can be atomized at the same time as the target analyte. Matrix effects arise when matrix components other than the target analyte react to form molecular species and sample background, thereby inhibiting formation of free target analyte atoms. GFAAS has been reported in analysis of Cd, Cr, Pb in brown sugar (Dos Santos, 2018), Pb in milk (de Oliveira, 2017), Zn in wine (Mollo, 2017), Cd, Cr, Cd in fruit juice (Anastácio, 2018), and Cd, Pb, Cr in yoghurt (de Andrade, 2018). According to literature GFAAS has not been reported on analysis of pet food samples.

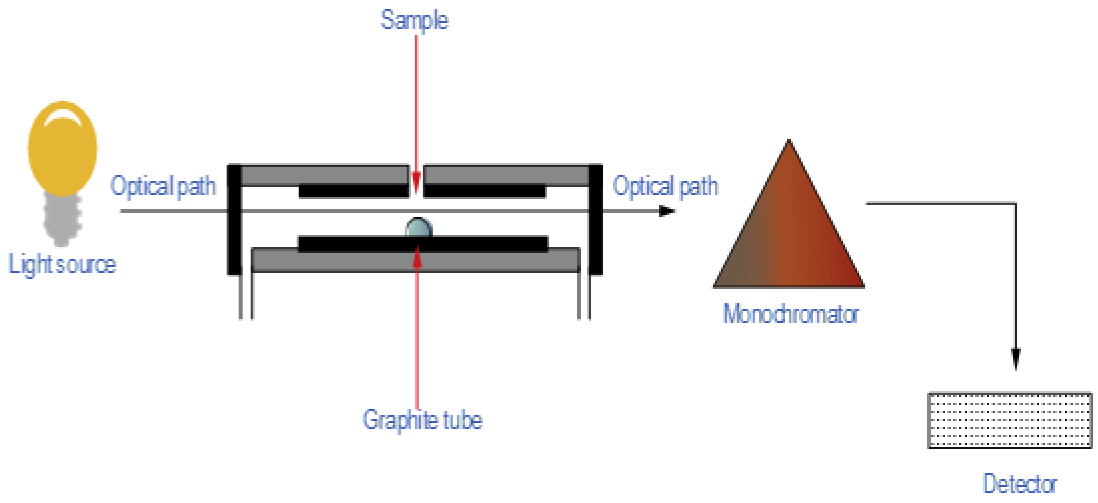

Figure 3.2: A schematic diagram of graphite furnace atomic absorption spectrometry (GFAAS) (Adapted from (Fairulnizal, 2019)).

3.1.3 Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES)

In the past decade, ICP-OES has gained popularity for elemental analysis, owing to its capabilities to simultaneously detect multi-elements (Bulska & Wagner, 2016; Douvris et al., 2023). It uses inductively coupled plasma to excite atoms and ions which emit characteristic wavelength of light, which is measured using an optical spectrometer (de la Guardia & Armenta, 2011), thus it uses emission spectra of elements for qualitative and quantitative purpose. As shown in **Fig. 3.3,** a sample is usually introduced in liquid form, dissolved, or digested in an acid and then nebulized to form a fine aerosol which is transported to the plasma. Sample preparation methods such as microwave acid assisted digestion, ultrasound acid assisted extraction, microwave acid assisted extraction, etc. are used for converting samples into liquid form. Like the ICP-MS, ICP-OES it is also known for its extremely low detection limits, exceptional precision, and accuracy (Šelih et al., 2014). The current biggest competitor for ICP-OES in elemental analysis is ICP-MS. ICP-MS has extremely lower detection limits (1000x) (Olesik, 2020), however as mentioned earlier, ICP-MS is more costly (instrument purchase, operational costs) and has severe matrix effects when compared to ICP-OES. These matrix effects may be divided into two categories: matrix induced signal intensity changes and matrix induced spectral overlap problems. ICP-OES has found routine utility in analysis of various food samples such as wine (Šelih et al., 2014), and tomatoes (Bressy et al., 2013).

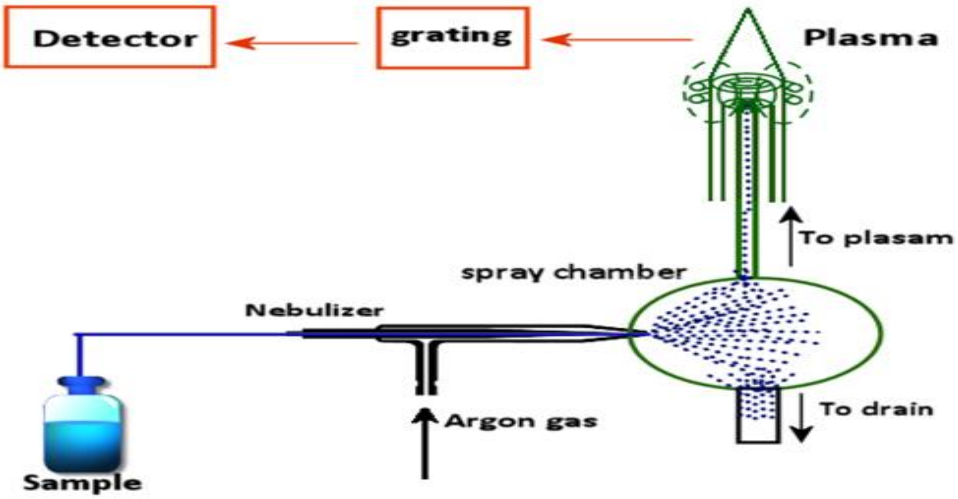

Figure 3.3: A schematic diagram of inductively coupled plasma-optical emission spectrometry (ICP-OES) (Adapted from (Udgave, 2017)).

3.1.4 Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

It is also one of the most sensitive mass spectroscopic techniques for detection and quantification of a wide range of elements at trace and ultra-trace levels (Wilschefski & Baxter, 2019). As shown in **Fig. 3.4** it consists of six components: sample introduction system, inductively coupled plasma (ICP), interface, ion optics, mass analyzer and detector (Wilschefski & Baxter, 2019). It uses argon plasma to atomize sample analyte into ions which are quantified and detected by a mass spectroscopy (MS) (Bulska & Wagner, 2016). ICP-MS is capable of multi-elemental detection and has a wide linear range (Agatemor & Beauchemin, 2011). Moreover ICP-MS has extremely low detection limits and has a high sample throughput like ICP-OES (Wilschefski & Baxter, 2019). ICP-MS has been reported for successfully detecting and quantifying trace and ultra-trace elements in various food samples such as complete dog and cat food (Davies *et a*l., 2017), dry dog food (Pereira *et al*., 2018), fresh meat (Han *et al*., 2022), rice, bean, egg, fish, bread, sugar, vegetables, cheese, powder milk, butter, wheat, pear, Brazilian nuts, coffee, chocolate, biscuits and pasta) (Elene P. Nardi *et al*., 2009), wheat flour(Alhagri & Albeshry, 2023), white rice (Lee *et al*., 2023), cocoa solids, and chocolate (Yanus *et al*., 2014), cocoa powder(Gianluigi Maria Lo Dico, 2018), and milk (Lee *et al*., 2022). ICP-MS weaknesses are its high operational costs, and the occurrence of spectral and non-spectral interference (Agatemor & Beauchemin, 2011). According to literature ICP-MS is one of the widely reported analytical techniques in food analysis, with its application peaking in the last decade.

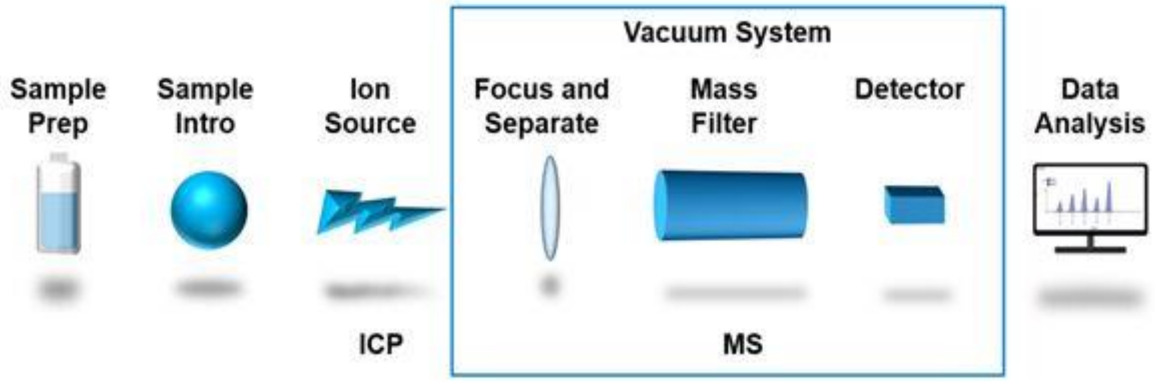

Figure 3.4: A schematic diagram of inductively coupled plasma-mass spectrometry (ICP-MS) (Adapted from (Košler J, 2003)).

3.1.5 Xray Fluorescence Spectroscopy (XRF)

It is a technique used for elemental analysis in both solid, liquid, and powdered food samples. XRF consists of two main components, the x-ray source and a detector (El Hosry, 2023; Frydrych, 2023) as shown in **Fig. 3.5(A).** XRF uses interaction of x-ray and the material of food samples to determine their elemental composition. X-rays pass through the sample material, and some of the x-rays are absorbed (Byers, 2019; Pashkova, 2018). The absorbed x-rays interact with the sample material at an atomic level, causing scattering and releasing of electrons, photons, and fluorescent x-rays as shown in **Fig. 3.5(B)** (El Hosry, 2023). X-ray has gained popularity in research owing to its non-destructive nature and ability to rapidly detect and fingerprint elements at low cost, as it does not require sophisticated apparatus for analysis compared to other techniques (Frydrych, 2023). It is also non-destructive in most circumstances and it's also a green analytical technique. It does not require sample dissolution or digestion; thus, it allows analysis of the sample without destroying it and altering its structure with solvents such as acids.

There are two XRF methods, namely: wavelength dispersive XRF (WDXRF) and energy dispersive XRF (EDXRF) (El Hosry, 2023; Pashkova, 2018). The difference in the principle of these techniques is that WDXRF physically separates the x-rays according to their x-rays, and EDXRF directly measures the different energies of the emitted x-ray of the sample matrix (Frydrych, 2023). Both these methods have their demerits and merits, and they have different ranges of detectable elements. WDXRF has a wider range of detectable elements, stretching from sodium to beryllium, while EDXRF can only detect elements from sodium to uranium. WDXRF is also known to accurately characterize complex samples owing to its higher resolution which reduces spectral overlaps. XRF has been reported in quantification of metals in food samples such as quantification of trace elements in cherry tomato fruit (Panebianco, 2022), trace elements in food additives (Gazulla, 2021), Pb in parsley (Byers, 2019), macro elements and trace elements in broth and sauce base products (Perring, 2018), minerals in infant milk (Papachristodoulou, 2018), macro and trace elements in pet food samples (Perring, 2017), Cu in lettuce (Sacristán, 2016), and trace elements in carrots, spinach, radish (Gallardo, 2016).

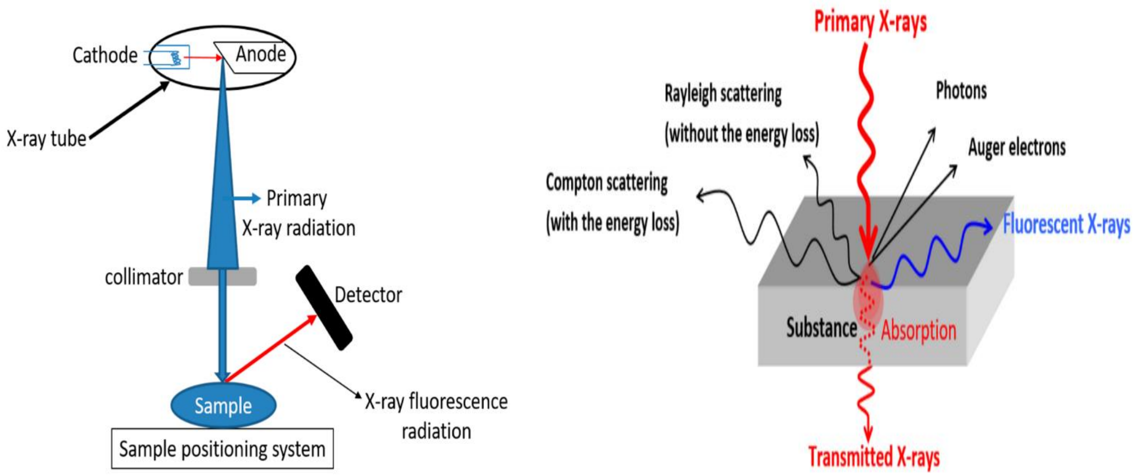

Figure. 3. 5: A schematic diagram of x-ray fluorescence spectroscopy (XRF) **(A)**, interaction of X-rays with substance (sample matrix) **(B)** (Adapted from ((El Hosry, 2023)).

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CHAPTER IV: COMPARISON OF ECO-FRIENDLY ULTRASOUND AND MICROWAVE BASED SAMPLE PREPARATION METHODS FOR SPECTROSCOPIC DETERMINATION OF HEAVY METALS IN PET FOOD SAMPLES

ABSTRACT

Microwave-assisted hydrogen peroxide digestion (MW-AHPD) and ultrasound-assisted hydrogen peroxide extraction (UA-HPE) methods were developed for the determination of Cd, As, Pb, Sn, and Cr in South African pet food samples by inductively coupled plasma-optical emission spectroscopic (ICP-OES). Multivariate tools, certified reference materials (wheat flour: FAP80467, fish muscle: ERMBB422) assessed the influential factors affecting both MW-AHPD and UA-HPE. The resulted optimum conditions for the MW-AHPD were: 200 °C microwave temperature, 40 mins digestion time, 0.5 g sample mass, and 5 mol/L H_2O_2 concentration. While UA-HPE was optimum at: 80 °C, 60 mins, 0.5g, 5 mol/L, for sonication temperature, extraction time, sample mass, and H_2O_2 concentration, respectively. The recoveries obtained for the MW-AHPD method ranged between 96-98% while UA-HPE recoveries were above 95%. Both MW-AHPD and UA-HPE demonstrated excellent reproducibility, $\leq$ 2.1% and $\leq$ 1.9 % respectively. Furthermore, both methods showed acceptable detection limits (MW-HPD: 0.0675 to 0.3765 µg/g and UA-HPE: 0.3498 and 0.49 µg/g) and greenness (MW-AHPD: 0.76 and UA-HPE: 0.74).

Application of MW-HPD in pet food samples gave element concentrations of 11.2-22.6, 6.4-11.9, 3.44-13.4, 0.44-2.98 and 0.18-0.98 µg/g, while UA-HPE gave 0.86-11.34, 4.50-11.45, 2.61-12.5, <DL-7.94, and <DL-1.04 µg/g, for Cd, Pb, As, Cr and Sn, respectively. However, three metals (Cd, Pb and As) were above the maximum tolerable limits for some of the fish and wheat based pet food according to the South African regulatory standard. The results showed that the two methods were adequate for determination of As, Cd, Pb, Sn, and Cr in pet food samples.

4.1 INTRODUCTION

The presence of heavy metals in pet food is a serious threat to animal health when it is above desirable levels (Macías-Montes *et al.*, 2021). Pet food is produced for domesticated animals to meet all nutritional needs and as complementary treats (Schleicher et al., 2019). The main raw materials for pets food include plant fats, cereals, grains, minerals, preservatives, flavorings, vitamins, seafood, meat, and animal derivates (Zafalon *et al.*, 2021). Pet foods can be grouped into; dry foods made by baking or extruding, wet food made by cooking and semi-moist food made by baking or extruding with a final moisture of 60 to 65% (Schleicher *et al.,* 2019*)* (White, 2023). It is assumed that throughout all the processing steps, measures are implemented to make sure the products are made from safe and quality ingredients. The main ingredients in pet food manufacturing are of plant and animal origin and these are susceptible to contamination with heavy metals due to natural bioaccumulation processes (Abd-Elhakim *et al.*, 2016; Zafalon *et al*., 2021). Most pet food is stored and transported in covered, lockable trucks at ambient temperatures to customers and distributors (Morelli *et al.*, 2021). Thus, present minimum risks to contamination.

Introduction of heavy metals such as Cu, Hg, Zn, Ag, Cd, Se, Ni, Pb, As, Sn into water sources leads to contamination of aquatic life and crops, which are the main ingredients in pet food manufacturing (Gunalan et al., 2018; Singh *et al.,* 2022). Several studies have reported presence of heavy metals in various ingredients which are commonly used in pet food formulation, namely, wheat flour (Alhagri & Albeshry, 2023), rice (Ebrahimi-Najafabadi et al., 2019), bovine tissues (muscle, liver, and kidney) (Esposito et al., 2019; Hashemi, 2018), and fish species (da Silva et al., 2021; Gu *et al*., 2016; Islam *et al*., 2015). Furthermore, several studies have reported presence of heavy metals in pet food (Abd-Elhakim *et al*., 2016; da Costa, 2013; Davies *et al*., 2017; Fernandes *et al*., 2018; Høgåsen *et al.*, 2016; Kazimierska *et al*., 2020; Kępińska-Pacelik *et al.*, 2023; Kim *et al*., 2018; Macías-Montes *et al.*, 2021; Pain *et al*., 2023; Pereira *et al.*, 2018; Squadrone *et al.*, 2017; Zafalon *et al*., 2021). The reported presence of heavy metals in pet food in the past decade has strengthened the well-known theory that ingestion of contaminated pet food is the main route exposing pets to toxic heavy metals (Abd-Elhakim *et al.*, 2016). Serpe and colleagues (2012), reported higher levels of mercury in kidneys of household dogs than stray dogs supporting the involvement of pet food in exposing pets to toxic heavy metals (Serpe et al., 2012). Several publications have reported presence of heavy metals in pets organs and blood, namely: hair (Rosendahl *et al.*, 2022; Sousa *et al*., 2013), blood (Rosendahl *et al*., 2022; Tomza-Marciniak et al., 2012), liver (Esposito *et al.*, 2019; Serpe, Russo, De Simone, *et al.*, 2012), and kidneys (Esposito *et al*., 2019; Serpe, Russo, De Simone, *et al.*, 2012). Continuous ingestion of pet food with high levels of heavy metals is associated with heavy metal poisoning which may lead to both acute and chronic health effects in pets. Cadmium (Cd) poisoning in pets affects all major organs, mainly the kidneys and liver leading to degeneration of renal tubule, renal atrophy, and renal functional failure (Council, 2006). Lead (Pb) poisoning causes acute health effects, namely vomiting, anorexia, and diarrhoea (Council, 2006; Høgåsen *et al*., 2016). Arsenic (As) poisoning affects the vascular system of pets, once arsenic enters the body it travels to the main organs, causing bleeding and swelling. Signs of acute arsenic poisoning are diarrhoea, vomiting, weakness, staggering, low body temperature, collapsing, and death (Garland & Garland, 2020; Gupta, 2018; Schmid *et al*., 2021). Chromium (Cr) exists in its trivalent and hexavalent forms; the hexavalent form is the most toxic to pets. There is limited evidence to support toxicity of chromium in pets, however chronic exposure to Cr leads to damage of the circulatory system, nerve tissues, kidney, and liver failure. Tin (Sn) is relatively low in toxicity, however if consumed in high concentration it may affect kidneys, liver, and pancreas. Subsequently, there is a crucial need for the development of rapid and effective methods for quantitative determination of heavy metals in pet food to ensure continuous monitoring of heavy metal concentration levels.

Inductively coupled plasma-optical emission spectroscopy (ICP-OES) is currently the most widely used atomic spectroscopic technique for ultra and trace elemental analysis in food samples due to its versatility, fast multi-elemental analysis capability, easily adjustable wavelength lines to obtain required sensitivity, high temperature of the plasma for refractory elements and easy operation (Khan *et al*., 2022). However, solid samples cannot be directly introduced into the plasma, they must either be transferred into the plasma using a solid accessory or they must be digested or extracted into an aqueous form. The latter is the most widely sample form used in plasma-based techniques (Bizzi *et al.*, 2017; Duyck *et al.*, 2022). Therefore, sample preparation methods such as extraction, and digestion are used to convert solid sample matrices using concentrated acids (pure or mixture). Amongst the variety of sample preparation methods to be considered such as wet digestion, we chose to explore microwave assisted digestion (MAD) and ultrasound-assisted extraction (UAE). MAD is commonly conducted by using concentrated acids (pure or in mixtures) in a closed vessel system, like microwave assisted digestion (MW-AD) (Khan *et al.*, 2022). Microwave based digestion methods are well-documented for food samples, because it is rapid, has high efficiency, safe to operate, and has lower risks of contamination and analyte losses (Alhagri & Albeshry, 2023b; Mohamed *et al*., 2020b; Mullapudi *et al.*, 2019).

The UAE is carried using a simple ultrasonic water bath or an ultrasonic probe (Dos Santos *et al.*, 2019b) using concentrated acids (pure or a mixture). Also, UAE has an advantage over other classical extraction methods extraction because it has better protection of sample matrices because of lower temperatures generated in the medium. According to literature, UAE has mainly been used for extraction of trace metals in biological sample matrices. It has been used for extracting essential and non-essential trace metals in carrot, reddish, cauliflower, pumpkin, and spinach, with recoveries between 96 % and 108.3%, and relative standard deviation below 2.61% (Ul-Haq *et al., 2021)*. In another study by Dos Santos and colleagues (2019), UAE was used for extraction of Cd and Pb from fresh apples, tomatoes, lettuce, and carrots, with recoveries of 97-112 %. In 2019 Dos Santos and colleagues (Dos Santos et al., 2019a)also extracted macro and micro elements in centrifugal sugar, with recoveries of 91-108%.

Despite both techniques' popularity and their application in several studies, like other wet chemistry sample preparation methods, UAE and MAD have a biggest challenge of using concentrated acids with oxidising properties which generate hazardous residues such as carcinogenic nitrous oxide (NO_x) during extraction(Kazi *et al.*, 2009; Villa *et al.*, 2015). To overcome the challenge of use of concentrated acids, greener microwave assisted hydrogen peroxide digestion (MW-AHPD) and ultrasound assisted hydrogen peroxide extraction (UA-HPE) were recently introduced as alternative sample preparation methods in food sample matrices, aiming at the use of dilute hydrogen peroxide. MW-AHPD and UA-HPE are regarded as greener sample preparation methods as it uses greener reagent H_2O_2, which converts to hydrogen, oxygen, and water at high temperatures of the ultrasonic bath. Furthermore, under heat, H_2O_2 dissociates to hydroxy radical (OH) attacking fatty acids, proteins, and carbohydrates in organic food samples. Dilute H_2O_2 also helps to eliminate plasma based spectral interference during ICP-OES elemental analysis. Recently, MW-AHPD was developed for spectroscopic determination of rare earth elements in coal samples (Zuma *et al.*, 2021), in milk powder (Muller *et al.*, 2016), carbohydrate-rich foods (honey, cassava, potato, corn and wheat flour) (Muller *et al*., 2017) and milk (whole, skimmed, and organic milk) (Park & Lee, 2022) for spectrometric determination of metal ions, while UA-HPE has also been developed before for extraction of multielement from sorghum flour(Curti et al., 2021).

The use of only H_2O_2 as a digestion solvent in microwave-assisted digestion and ultrasound-assisted extraction of food samples is scarce in the literature, however a lot of studies have been reported on the use of the combination of H_2O_2 and other acids in microwave assisted digestion (Alhagri & Albeshry, 2023a; Davies *et al*., 2017; Lee *et al*., 2022; Lemos *et al*., 2019; Mohamed *et al.*, 2020a; Mullapudi *et al*., 2019; Pereira *et al*., 2018; Shariff *et al.*, 2018; Zafalon *et al.*, 2021) and ultrasound assisted extraction (Lučić *et al*., 2022; Pereira *et al*., 2021; Ul-Haq *et al*., 2021),(Costa *et al*., 2020; Kazi *et al.*, 2009) .Therefore, the aim of the study was to develop a MW-AHPD and UA-HPE methods followed by ICP-OES analysis for determination of selected heavy metal ions (Cd, Pb, As, Sn and Cr) in South African pet food samples. The performance of the developed MW-AHPD and UA-HPE methods were measured by analysing food certified reference materials (CRMs). Furthermore, the obtained concentration levels were compared with the limits of various regulatory bodies to assess if the desired limits were exceeded. According to the authors' knowledge, it is the first time that MW-AHPD and UA-HPE methods are multivariate optimized for determination of heavy metals in pet food by ICP-OES.

4.2 EXPERIMENTAL SECTION

4.2.1 Materials and Chemicals

The pet food samples were divided into 5 groups according to the ingredients of interest (fish, wheat, maize, brown rice, and leafy green vegetables) as shown in **Table 4.1**. Both brands of the dry pet food samples (FBA, WBA, MBA, BRBA, LGVBA, FBB, WBB, MBB, BRBB, LGVBB) were purchased from the local supermarkets in Florida Park, Johannesburg, South Africa. All the samples were grounded into fine powder using a pre-cleaned mortar and pestle and homogenized prior to extraction. The mortar and pestle were thoroughly acid washed before using them for a new sample to prevent contamination. Ultrapure deionized water with resistivity of 18.2 MΩ cm was obtained by using Millipore RiOs 5 reverse osmosis and a Millipore Milli-Q academic deionizer system (Millipore, Bedford, MA, USA). This highly purified water was used throughout the experiments for preparation of solutions and rinsing of glassware. It is worthy to mention that microwave polytetrafluoroethylene-Teflon (PTFE-TFM) vessels, glassware and inductively coupled plasma-optical emission spectroscopic (ICP-OES) sample tubes were previously decontaminated by washing with laboratory detergent soapy solution and soaking in 5% (v/v) HNO_3 solution for at least 24 hours, before rinsing with deionized water. Thereafter, the glassware was allowed to dry in an oven (EcoTherm Labotec) for overnight.

Suprapur nitric acid (65%: v/v) and hydrogen peroxide (30%: v/v) were purchased from Merck, Johannesburg, South Africa. Multielement analytical standard solutions in the range of 0.1 to 5 mg/L were prepared by appropriate dilutions of respective single element (As, Pb, Cr, Sn and Cd) stock standards solutions containing 100 mg/L (Sigma-Aldrich, Johannesburg, South Africa) with 3 % HNO_3. Validation experiments for the proposed MW-AHPD and UA-HPE were performed by using certified reference materials (CRMs), which were wheat flour (FAP80467) and fish muscle (ERMBB422) form Sigma-Aldrich, Johannesburg, South Africa. These CRMs were used without any further preparation (Pereira *et al.*, 2018). Polyvinylidene difluoride (PVDF) microfilters for filtration of the samples prior to ICP-OES samples and the weighing boats were also purchased from Sigma-Aldrich, South Africa.

Table 4.1: Grouping of the pet food samples from two brands.

Brand A	**Brand B**
Fish brand A (**FBA**)	Fish brand B (**FBB**)
Wheat brand A (**WBA**)	Wheat brand B (**WBB**)
Maize brand A (**MBA**)	Maize brand B (**MBB**)
Brown rice brand A (**BRBA**)	Brown rice brand B (**BRBB**)
Leafy green vegetable brand A (**LGVBA**)	Leafy green vegetable brand B (**LGVBB**)

4.2.2 Instrumentation

Microwave digestion system.

A closed-vessel microwave digester, Multiwave 5000 (Anton Paar Southern Africa (Pty) Ltd., Midrand, South Africa), equipped with a 20SVT50 rotor system, high temperature polytetrafluoroethylene-Teflon (PTFE-TFM) vessels with 100 mL capacity and 1800 W maximum microwave power at 220 ^{o}C was used for all the digestion experiments of selected pet food samples. Each 100 mL PTFE vessel is composed of fluoropolymer, to accommodate volume ranges from 10 to 100 mL. There by allowing rapid, effective, and rapid digestion experiments with minimal losses of volatile elements and low detection limits. Multiwave 5000 internal pressure and temperature were continuously monitored by a control vessel fitted with pressure and temperature sensors. To ensure no outlier digestion reactions, an external IR sensor was put in place to monitor the temperature of each standard PTFE vessel.

Ultrasound extraction system.

An ultrasound device applied in the extraction method was Scientec 702 4.0 L Ultrasonic bath (Scientific Engineering, South Africa) with a power of 100 W, equipped with a timer function (max 60 mins), heating element maximum capacity 80 ^{o}C, stainless steel basket and lid. The water bath was filled with 3.0 L of ultrapure water. Ultrasonic radiation (value) was applied to pet food samples-solvent mixture in 50 ml polystyrene centrifuge tubes through the water bath.

Inductively coupled plasma - optical emission spectrometry

The concentration levels of As, Pb, Cd, Cr and Sn in microwave digests were determined by using Agilent ICP-OES 700 Series instrument (Agilent Technologies, Santa Clara, CA, USA), equipped with auto-sampler AS 93-plus, concentric nebulizer, cyclonic spray chamber and axial torch. Argon with a minimum purity of 99.95% was used as main auxiliary, and nebulizer gas (Afrox, South Africa). The instrumentation operating conditions were set to 1200 W RF power, 15 L/mins plasma gas stream, 1.5 L/mins auxiliary gas stream, 0.75 L/min nebulizer gas stream, 85 rpm pump speed, 15 s sample up-take delay and stabilization time. An axial geometric torch view, 2-point background correction and three repeats were utilized to generate meaningful metal ion detection. Most prominent emission wavelength lines liberated from spectra impedance were 384.123, 245.231, 205.336, 204.784, and 325.457 nm for Cd, Pb As, Cr and Sn, respectively.

4.2.3 Microwave-assisted hydrogen peroxide digestion procedure.

An optimum amount of homogenized powdered pet food sample was weighed using weighing paper and transferred into polytetrafluoroethylene-Teflon (PTFE-TFM) vessels. Thereafter, an accurate volume of 15 mL dilute H_2O_2 was introduced into the same vessel. The pre-digestion reactions were allowed to proceed for 20 mins (Chen et al., 2015; da Costa, 2013), then the vessels were tightly closed and inserted to the 20SVT50 rotor. The rotor was subjected to Multiwave 5000 radiation system at optimum conditions (temperature: 200°C, digestion time: 40 mins). After completion of the digestion, the vessels were taken out of the microwave and were allowed to cool down to room temperature (25 ^{o}C) for 20 mins (Mketo, 2015; Soós Á, 2021). The digests produced were quantitively transferred into 25 mL volumetric flasks and the flasks were then filled up to mark with ultrapure water. The aqueous solutions were then passed through polyvinylidene difluoride (PVDF) microfilters, prior to metal analysis by ICP-OES. Each sample was digested in triplicates (n=3) with a blank (dilute H_2O_2) as the fourth sample.

4.2.4 Ultrasound-assisted hydrogen peroxide extraction procedure.

Ultrasound-assisted hydrogen peroxide extraction was carried out in an ultrasonic water bath device (Scientec 702 4.0 L) at a constant power of 100 W and frequency of 50 kHz. An optimized amount of homogenized powdered pet food sample was weighed using weighing paper and immersed into a self-standing 50 ml polystyrene centrifuge tube containing 15 mL dilute H_2O_2. The centrifuge tube was then immersed in an ultrasonic water bath for extraction at optimum conditions (temperature and time). After completion of the extraction process, the tube containing the extract was taken out of the water bath and allowed to cool down to ambient temperature (25 ^oC) for 15 mins. The extract produced was quantitively transferred into 25ml volumetric flask and the flask was filled up to calibration mark with ultrapure water. The aqueous solutions were then filtered with polyvinylidene difluoride (PVDF) microfilters prior to analysis by ICP-OES. All the ultrasonic extractions were carried out in triplicates (n=3) with a reagent blank (5mol/L H_2O_2) as fourth sample.

4.2.5 Multivariate optimization procedure for the proposed MW-AHPD.

To optimize the most influential parameters (digestion time, microwave temperature, sample amount and H_2O_2 concentration) affecting the efficiency of the proposed MW-AHPD method, a 2-level full factorial design (2^4) and response surface methodology (RSM) mathematical tools were used. These two models are well-documented for the optimization of four parameters (Pereira R. M., 2019) and were interpreted by Minitab 18 data analysis software. The 2-level full factorial design (2^4) was mainly for screening purposes to eliminate insignificant parameters and RSM was for further multivariate optimization of the significant parameters. Factors and levels that were investigated for the first order 2-level full factorial design (2^4) are shown in **Table 4.2.**

The most significant factors were assessed by using Pareto charts and were further optimized by RSM (see **Table 4.3**).

Table 4.2: Factors and levels that were used for first order 2^4 full factorial designs.

Variable (Factor optimized)	Low level (-)	High level (+)
Sample mass (g)	0.2	0.5
H_2O_2 concentration (M)	1	5
Digestion time (minutes)	10	30
Microwave Temperature (°C)	80	200

Table 4.3: Factors and levels that were used for the second order response surface methodology.

Variable (Factor optimized)	Low level (-)	High level (+)
Digestion time (minutes)	20	40
Microwave Temperature (°C)	90	200

4.2.6 Multivariate optimization procedure for the proposed UA-AHPE

The effect and interaction of all parameters (sample mass, extraction time, extraction temperature and concentration of H_2O_2) on extraction of As, Pb, Cd, Cr and Sn from pet food samples was determined using a 2-level full factorial design (2^4) and response surface methodology (RSM) chemometric tools. Parameters and levels that were investigated for the first order 2-level full factorial design (2^4) are shown in **Table 4.4.** The most significant parameters were assessed by using Pareto charts and were further optimized by RSM (see **Table 4.5**). Response surface methodology (RSM) was used for further investigation of the effect of significant parameters (extraction time and extraction temperature) on efficiency of extraction (response) of As, Pb, Cd, Cr and Sn. The effects of extraction time and temperature on efficiency of extraction (response surface) were viewed as 3D surface plots.

Table 4.4: Factors and levels that were used for first order 2^4 full factorial designs.

Variable (Factor optimized)	Minimum level (-)	Maximum level (+)
Sample mass (g)	0.1	0.2
H_2O_2 concentration (M)	1	5
Sonication time (minutes)	20	60
Sonication Temperature (°C)	30	80

Table 4.5: Factors and levels that were used for the second order response surface methodology.

Variable (Factor optimized)	min level (-)	max level (+)
Sonication time (minutes)	30	60
Sonication Temperature (°C)	40	80

4.2.7 Analytical performance of the MW-AHPD and UA-HPE

The performance of the proposed MW-AHPD and UA-HPE methods were assessed by digesting and extracting food CRMs (wheat flour: FAP80467, fish muscle: ERMBB422) containing known amount of the targeted analytes (Cd and As) followed by ICP-OES analysis of the heavy metals in the digest. Thereafter, accuracy, precision (repeatability and reproducibility), limit of detection (LOD), limit of quantification (LOQ) and correlation coefficients, determined (Alhagri & Albeshry, 2023a; Mdluli *et al.*, 2023). The accuracy of the MW-AHPD and UA-HPE methods was reported in terms of percentage recoveries of each metal (Cd, Pb, As, Cr, and Sn) and the recoveries were calculated using **equation 4.1**.

$$\%R = \frac{Experimental\ value}{CRM\ value} \times 100\% \qquad \textbf{Eq.4. 1}$$

where the CRM value is the certified concentration value of a particular metal in fish and wheat flour certificates, whilst experimental value is the value obtained from the ICP-OES measurement incorporated with the dilution factor.

Precision defined as the relative standard deviation (RSD%) of the proposed MW-AHPD and UA-HPE methods was determined by analysis of 7 independent certified reference materials. Precision was estimated from the relative standard deviation (RSD%). The limits of detection (LOD) and limits of quantification (LOQ) were determined by measuring 20 blank solutions and were defined according to IUPAC definition, with the lowest concentration of an analyte giving signals equal to three or ten times, respectively, multiply by the standard deviation (SD) of blank signal divided by the slope of the calibration curve as shown in **equations 5.2** and **5.3**. The method detection limits (MDL) and the method quantification limit (MQL) were calculated by multiplying the obtained LOD and LOQ with the dilution factor of the proposed methods, respectively (see **equations 4.4** and **4.5**

$$Limit\ of\ detecton\ (LOD) = \frac{3 * SD}{Method\ calibration\ slope} \qquad \textbf{Eq.4. 2}$$

$$Limit\ of\ quantification\ (LOQ) = \frac{10 * SD}{Method\ calibration\ slope} \qquad \textbf{Eq.4. 3}$$

$$Method\ detection\ limit\ (MDL) = \frac{LOD * Final\ volume}{Optimum\ mass} \qquad \textbf{Eq.4. 4}$$

$$Method\ quantification\ limit\ (MDL) = \frac{LOD * Final\ volume}{Optimum\ mass} \qquad \textbf{Eq.4. 5}$$

4.3 RESULTS AND DISCUSSION

4.3.1 Two level factorial design optimization of MW-AHPD

A two-level full factorial design (2^4) was used to determine the effect of microwave temperature, A (80 to 200 ^{o}C), digestion time, B (10-30 mins), sample mass, C (0.2 to 0.5 g) and H_2O_2 concentration, D (1-5 mol/L) and their interactions during MW-AHPD process. The factorial design was used to test all possible combinations of these factors, by varying all factors at different levels and measuring their effects on the MW-AHPD process. The obtained results from the model were later visualized using Pareto charts as shown in **Fig. 4.1**. The latter demonstrated that for both Cd and As, microwave temperature and digestion time were the most significant factors at 95 % confidence level during the MW-AHPD method. However, sample mass and H_2O_2 concentration were observed to be statistically insignificant as shown in **Fig. 4.1**. It is worth noting that sample mass 0.5 g was chosen for this digestion method as 0.2 g was not giving good recoveries compared to 0.5 g. However, increasing sample mass beyond 0.5 g did not show much significant changes in terms of the recoveries obtained. The concentration of hydrogen peroxide (5 mol/L) was also chosen for similar reasons as the sample mass, however at low concentration of hydrogen peroxide (1 mol/L) there was clear evidence of incompletion digestion when looking at the resultant digests, the ones at high concentration were clear versus the ones at low concentrations which were a bit cloudy. Increasing concentration of the hydrogen peroxide did not have a significant increase of recoveries. As a result, sample mass and H_2O_2 concentration were kept at 0.5 g and 5 mol/L, respectively during further optimization of digestion temperature and digestion time. Further optimization of microwave temperature and digestion time was also reported by other authors who performed digestion for determination of metals in food samples (Alhagri & Albeshry, 2023a; Mohamed *et al.*, 2020a; Rodrigo Mendes Pereira, 2019).

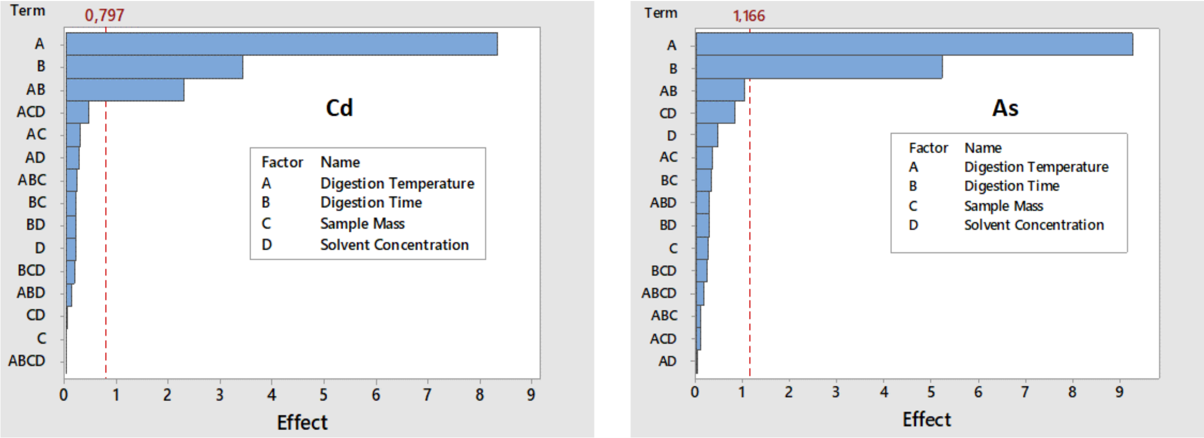

Figure 4.1: Pareto chart of main effects obtained from 2-level full factorial designs for both Cd and As. The vertical line defines the 95 % confidence interval [A=Temperature (°C), B=Time (mins), C= Sample Mass (g), and D=H_2O_2 concentration (M)]

4.3.2 Two level factorial design optimization of U-AHPE

Optimization of the developed UA-HPE method was done in 2 steps. In the first step, a two-level full factorial experimental design (2^4) was used to ascertain the effect and interaction between variables, at different levels. Pareto charts were used to visualize the results obtained from the 16 full factorial design experiments as shown in **Fig. 4.2**. The pareto charts showed the most significant parameters on the UA-HPE process, sonication time and sonication temperature were the most significant variables in both charts. The vertical line which defines 95 % confidence interval, demonstrating that these variables are statistically significant at 5 % confidence level for the UA-HPE process. However, sample mass and H_2O_2 concentration were statistically insignificant- 0.1g was chosen for this extraction process as masses greater than 0.1g were not giving good recoveries and there was incomplete extraction, evidenced by solid particles remaining in the extracts. The effects of concentration of the solvent, H_2O_2 below 5mol/L showed incomplete extraction and low recoveries whilst above 5 mol/L showed complete digestion but with no increase in recoveries. As a result of the above, sample mass and H_2O_2 were kept constant at 0.1g and 5 mol/L respectively during further optimization of the statistically significant factors, namely, sonication time and sonication temperature. Several studies have also reported further optimization of sonication time and temperature during ultrasound assisted extraction of metals in various food sample matrices, pepper (Lučić et al., 2022), guarana (Farias *et al.*, 2022), fruits and vegetables (Pereira *et al.*, 2021), vegetables (Ul-Haq *et al.*, 2021), bee pollen (Pohl *et al.*, 2020), pepper (Gamela *et al.*, 2020), chocolate bars(Villa *et al.*, 2015), green and black peas (Szymczycha-Madeja *et al.*, 2015), and bean seeds (Santos *et al*., 2009).

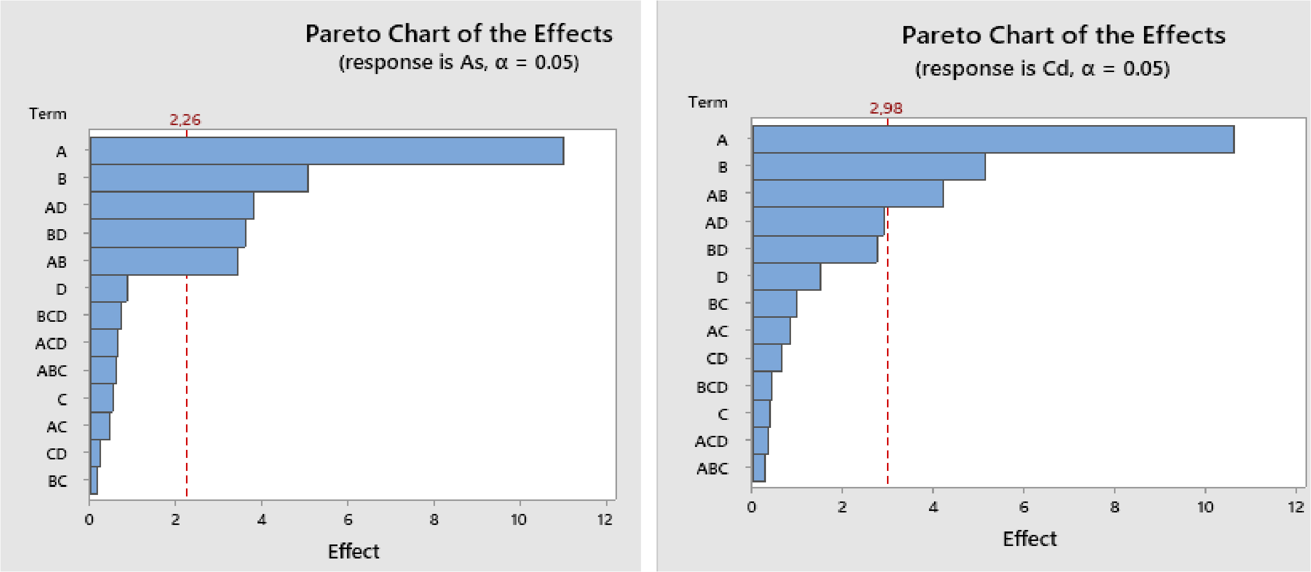

Figure 4. 2: Pareto chart of main effects obtained from 2-level full factorial designs for both Cd and As. The vertical line defines the 95 % confidence interval [A=Temperature (°C), B=Time (mins), C= Sample Mass (g), and D=H_2O_2 concentration (M)]

4.3.3 Response surface methodology optimization of MW-AHPD

Further determination of the effects of digestion time (20-40 mins) and microwave temperature (90-200 ^{o}C) in the MW-AHPD method was carried out using response surface methodology optimization technique (RSM). The RSM is a chemometric tool that helps establish quadric models to assist in determination of optimum conditions of an analytical method. The selection of the new optimum conditions ranges for further optimization of temperature and time was guided by the high recoveries given by the various combination of increased digestion time and digestion temperature. At temperatures below 90 °C we observed that some of the resulting digests were cloudy. We also observed incomplete digestion at digestion time less than 20 mins. It is also worth to note that various combinations of time (above 40 mins) and digestion temperature (200 °C) above did not show significant effect on obtained recoveries. Additionally, the optimum range selection was guided by the data generated by the analysis of variance statistical data obtained from RSM.

The response, i.e., digestion of Cd and As was computed using regression quadratic equations **4.11** and **4.12**, where A and B represent digestion time and digestion temperature. As shown in the regression equations, the significance of digestion temperature and digestion time affecting digestion of both Cd and As was in the following order: A, B, A*B. In general, the increase of both digestion temperature and digestion time resulted in an increase of digestion of both metals. To analyse the effect of the combined digestion temperature and digestion time, graphical representation of the regression equation i.e., three-dimensional response surface plots were produced and used for the visualization of the statistical data generated by the RSM (**see Fig. 4.3**). Based on the surface plots and regression quadratic equations, the maximum analytical response for both Cd and As were achieved at 200 °C and 40 mins for temperature and time, respectively. The newly developed MW-AHPD method's optimum conditions were compared with other previously developed microwave assisted studies which were performed using only pure H_2O_2 (without acids, e.g., HNO_3) as a digestion solution. It was observed that all the literature reported MW-AHPD methods utilized higher microwave digestion temperatures (250 °C) for digestion time less than 30 mins using higher concentrations of H_2O_2, 50% w/w^{-1} H_2O_2 in milk (Muller et al., 2016), 50% w/w^{-1} H_2O_2 in flour and honey (Muller et al., 2017) and 30% w/w^{-1} H_2O_2 in milk(Park & Lee, 2022). The current study utilized a more dilute H_2O_2 (5M) at 200 °C for 40 minutes.

$$Cd = 73.106 + 0.26878A + 0.2961B - 0.002928A * B \qquad \textbf{Eq.4. 6}$$

$$As = 75.457 + 0.2298A + 0.2478B - 0.002282A * B \qquad \textbf{Eq.4. 7}$$

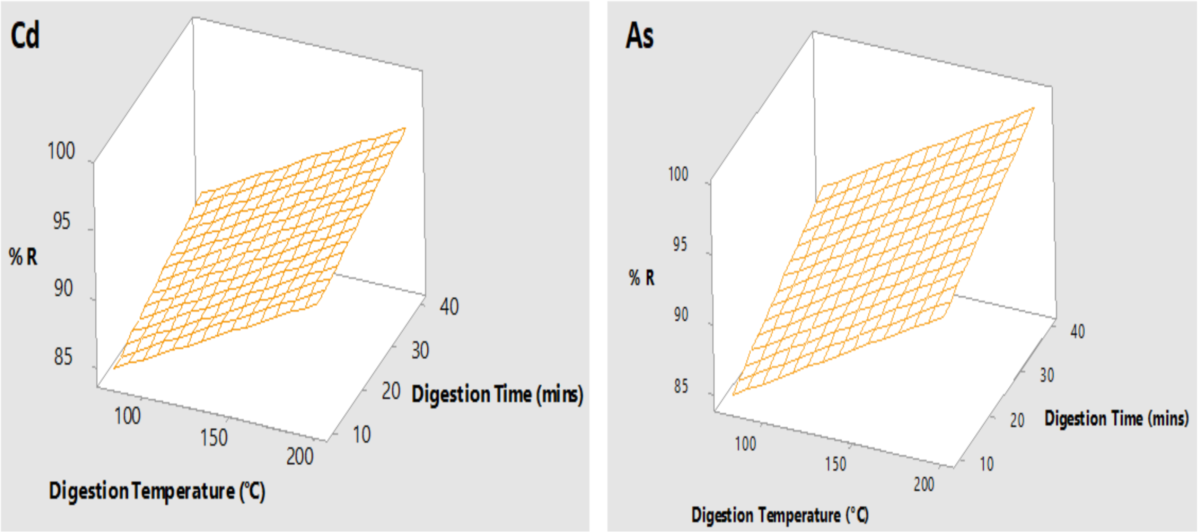

Figure 4.3: Response surface plots for percentage recoveries of Cd and As, as a function of microwave temperature(°C), and digestion time (min) at a constant sample mass (0.5 g) andH_2O_2 concentration (5 mol/L).

4.3.4 Response Surface methodology optimization of UA-HPE

The statistically significant t variables namely sonication temperature (40-80°C) and sonication time (45-60 mins) were further optimized using RSM to maximize the extraction yield of UA-HPE method. Generally, the temperatures and time variables were directly proportional as shown graphically in **Fig 4.4**, Additionally, the selection of the optimum range variables was guided by data generated by the analysis of variance statistical data obtained from RSM tool. The response, i.e., extraction of Cd and As was modelled using a regression quadratic equations **4.13** and **4.14**, where A and B represent sonication time and temperature. As shown in the regression quadratic equations, the significance of sonication time and sonication temperature on extraction of both Cd and As was in the following order: A, B, A*B. Based on the generated regression quadratic equations and the 3-dimensional surface plots, the maximum analytical response for both metals was achieved at 80 °C and 60 mins for sonication time and sonication temperature, respectively.

$$Cd = 61.214 + 0.34152A + 0.2344B - 0.003188A * B$$ **Eq.4. 8**

$$As = 76.029 + 0.23107A + 0.27422B - 0.002499A * B$$ **Eq.4. 9**

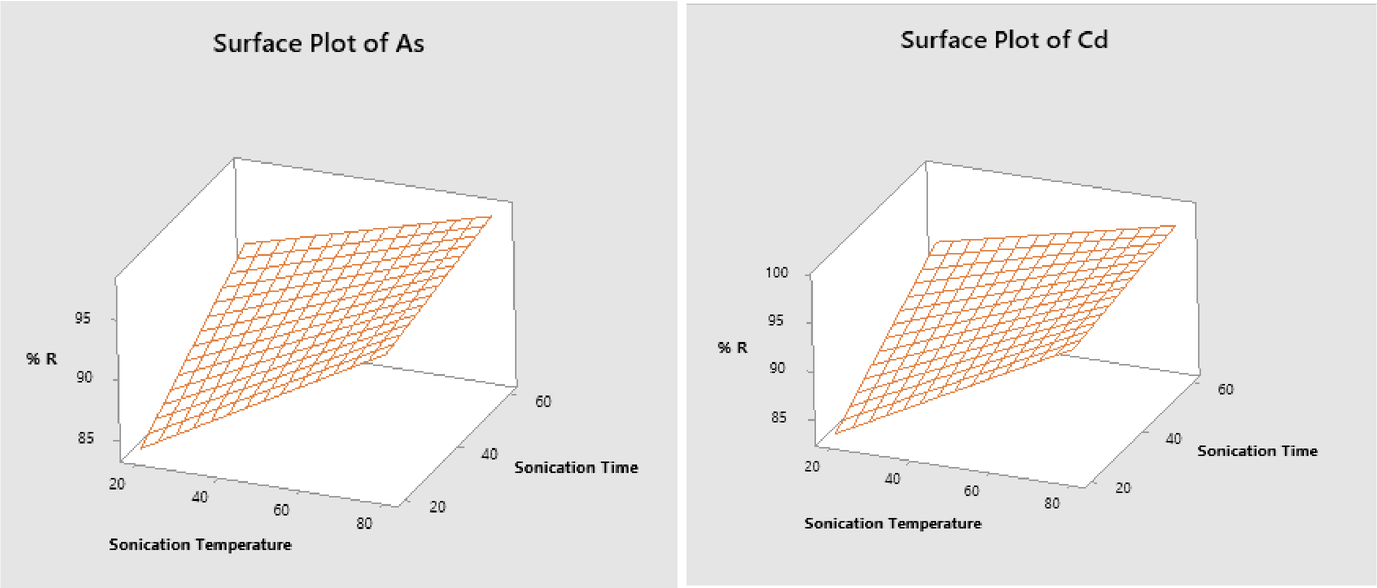

Figure 4. 4: Response surface plots for percentage recoveries of Cd and As, as a function of sonication temperature(°C), and sonication time (min) at a constant sample mass (0.1g) and H_2O_2 concentration (5 mol/L).

4.3.5 Validation of the proposed MW-AHPD and U-AHPE methods

The validation results of the proposed MW-AHPD and UA-HPE methods are presented in **Table 4.6 and Table 4.7**. The analytical figures of merit investigated were standard deviation of the calibration curve (SD), sensitivity (slope of the calibration curve), correlation coefficient (r^2), method limit of detection (MLD), method limit of quantification (MLQ), accuracy and precision. The proposed MW-AHPD method demonstrated good sensitivity for Cd (5.88 x 10^3 cps L mg^{-1}) and As (1.15 x10^3 cps L mg^{-1}), with accepted correlation coefficients of 0.9945 and 0.9923, respectively. These results were also confirmed by the low method of detection limits observed (0.0675 and 0.3765 µg/g). Furthermore, the validation results demonstrated that the MW-AHPD was precise (1.8 to 2.8 %) and accurate (98.1 to 98.9 %) for both As and Cd in white flour CRM (FAP80467). In the case of UA-HPE method, it also demonstrated good linearity with correlation coefficients greater than 0.99 for both Cd and As. Furthermore, the method demonstrated excellent sensitivity for both Cd (4.2575 x 10^3 cps L mg^{-1}) and As (3.2411 x10^3 cps L mg^{-1}) and low method detection limits (0.3498 and 0.49 µg/g). The low method of detection limits indicates that the newly developed UA-HPE has high sensitivity as well. The accuracy (>95%) of the method was validated using a certified fish muscle reference material (ERMBB422), and there was no significant difference between the certified values and those obtained using the newly developed UA-HPE.

The newly developed MW-AHPD and UA-HPE methods were compared with other digestion and extraction methods that were used in determination of different metal ions in various food samples as shown in **Table 4.8 and 4.9.** The results in **Table 4.8** show that the recoveries (more than 98 %) of the current study MW-AHPD method were in line with the ones reported in literature. While the other studies may have obtained recoveries like the current study it is worth noting that the newly developed method used only dilute H_2O_2 (5 mol/L), whereas other studies used concentrated H_2O_2 (9.86 mol/L) in combination with other concentrated acids as digesting solvents. Concentrated acids cause environmental pollution when discarded after the digestion (Bizzi, et al., 2017). Furthermore, some of these acids are corrosive in nature and might affect the glassware used in the laboratory and the optics for the spectroscopic techniques. Furthermore, this method was observed to be highly reproducible (≤ 2.1%) for both intra and inter-day repeatability. Most reported methods did not report reproducibility. However, the work that reported water bath acid digestion for determination of Cd, Pb Fe Zn and Cu in fish samples by flame-atomic absorption spectroscopy (FAAS) showed relatively poor precision (4-9%) as compared to the

MW-AHPD method. The digestion time (40 min) was also comparable with the literature report. This implies that the newly developed MW-AHPD method is precise, accurate, sensitive, eco-friendly, and rapid as compared to the latest digestion methods reported in literature. Therefore, this newly developed MW-AHPD method can be used as a standard method for quick monitoring of heavy metals in pet food samples.

In the case of newly developed UA-HPE method recoveries (>95%) obtained in this work were in line with recoveries reported in literature as shown in **Table 4.9.** While other previously reported studies obtained recoveries like this work, it is worth noting that this work also used greener reagent (dil.H_2O_2) give concentration used with an insignificant amount of HNO_3 (1ml, 70 wt %), whereas other extraction studies used concentrated H_2O_2 in combination with other larger amounts of other concentrated acids. Concentrated acids have several limitations as mentioned above. When compared with other reported studies in **Table.4.9**, this method demonstrated to be highly reproducible (≤ 1.84%) for both intra and inter-day repeatability. (Ul-Haq et al., 2021) also reported precision (≤ 2.61%), close to this work when using a combination of diluted HNO_3 (0.5 mol/L) and H_2O_2 (10%) in extraction of non-essential and essential metals in vegetables. (Curti et al., 2021) reported poor precision (≤7.7%) in ultrasound assisted extraction of metal ions from sorghum flour even though he used 7 mol/L of HNO_2 combined with H_2O_2. Extraction time in this current work (60 mins) was also comparable with that of literature (11-60 mins). This implies that the newly developed UA-HPE method is greener, accurate, sensitive, and rapid when compared to recently reported studies. Therefore, this method, may be used for quick monitoring heavy metals in pet food and other solid samples.

Table 4.6: Analytical features of the MW-AHPD method for quantitative extraction of Cd and As in wheat flour CRM (FAP80467): Experimental conditions (sample amount, 0.5g; H_2O_2 concentration, 5 mol/L; microwave temperature, 200 °C; digestion time, 40 mins and n=3)

Metal	SD of blank	Sensitivity (cps L mg^{-}	Accuracy	Precision	LOD	LOQ	MDL	MQL
Cd	2.6547	5.88 x 10^3	98.9	2.1	1.35	4.514	0.0675	0.2257
As	2.889	1.15 x 10^3	98.1	1.8	7.53	25.12	0.3768	1.256

Table 4.7: Analytical features of the UA-HPE method for quantitative extraction of Cd and As in fish muscle CRM (ERMBB422): Experimental conditions (sample amount, 0.1g; H_2O_2 concentration, 5 mol/L; sonication temperature, 80 °C; sonication time, 60 mins and n=3)

Metal	SD of blank	Sensitivity (cps L mg^{-1})	Accuracy (%)	Precision (%)	LOD (µg/L)	LOQ (µg/L)	MDL (µg/g)	MQL (µg/g)
Cd	1.9856	4.2578 x 10^3	95.55	1.9	1.399	4.6634	0.3498	1.1659
As	2.1224	3.2411 x 10^3	95.81	1.84	1.96	6.548	0.49	1.637

Table 4.8: Comparison of accuracy (%), MDL (ng/g) and precision achieved (% RSD) achieved by MA-HPD with literature studies.

Sample Matrix	**Sample preparation Method**	**Reagent**	**Reagent volume (mL)**	**Metal ion**	**Detection technique**	**Time (mins)**	**MDL (µg/g)**	**Accuracy (%)**	**Precision (%)**	**Ref.**
Dry Pet foods	MW-AHPD[a]	Dilute H_2O_2(5 mol/L)	15	Cd, Pb As, Cr, and Sn	ICP-OES[d]	40	0.0675 0.3768	>98	< 2	This work
Wheat flour	M-AAD[b]	65% HNO_3 & H_2O_2 (8 mol/L)	12	Mg, K, Ca, Al, Mn, Fe, Cu, Zn, Se, Rb, V, Cd, As	ICP-MS[e]	60	0.00001-0.207	90.43-109.76	0.42-4.89	(Alhagri & Albeshry, 2023a)

Wet and dry pet foods	Wet digestion	65% HNO_3 & 30% H_2O_2	8	Cd, Cr, Pb, Sn	F-AAS[f]	-	-	94-98	-	(Abd-Elhakim et al., 2016)
Dog and cat foods	M-AAD	65% HNO_3 & 30% H_2O_2	3.5	Sb, Ba, Cr, Sn, Fe, Hg	ICP-OES	30	-	-	-	(Zafalon et al., 2021)
Dog and cat foods	M-AAD	68% HNO_3 & 30% H_2O_2	5	Various Minerals	ICP-MS	45	-	>95	-	(Davies et al., 2017)
Fish	WB-AD[c]	HNO_3 & $HClO_4$	10	Cd, Pb Fe Zn Cu	F-AAS	300	0.2-1.125	84-89	4-9	(Malhat, 2011)

Notes: [a]Microwave assisted-hydrogen peroxide digestion, [b]microwave assisted-acid digestion, [c]water bath-acid digestion, [d]inductively coupled plasma-optical emission spectroscopy, [e]inductively coupled plasma-mass spectrometry, [f]flame atomic absorption spectroscopy

Table 4.9: Comparison of accuracy (%), MDL (ng/g) and precision achieved (% RSD) achieved by UA-HPE with literature studies.

Sample Matrix	Sample preparation Method	Reagent	Reagent volume (mL)	Metal ion	Detection technique	Time (mins)	MDL (µg/g)	Accuracy (%)	Precision (%)	Ref.
Dry Pet foods	UA-HPE[a]	Dil H_2O_2(5 mol/L)	15	Cd, Pb As, Cr, and Sn	ICP-OES[b]	60	0.35 0.49	>95	< 1.84	This work
Vegetables	UAD[b]	HNO_3 (0.5 M)-H_2O_2 (10 %)	5	Al, Cr, As, Cd, Pb, Mn, Fe, Cu, and Zn	FAAS[c] and ICP-OES.	11	-	96-108.3	< 2.61	(Ul-Haq et al., 2021)
Vegetables and fruits	UAD[b]	2.5 mol L^{-1} HNO_3)	20	Cd, and Pb	[d]ETAAS	15	-	112 ± 7/97 ± 9	-	(Pereira et al., 2021)

Sorghum flour	UAD[b]	HNO_3, 7 mol L^{-1}, and H_2O_2	6	Cu, K, Mg, Mn, P, and Zn	[c]MIP OES	30	0.6 to 89	-	≤7.7%	(Curti et al., 2021)
Centrifugal sugar	UAD[b]	60:40% (v/v) of HNO_3:H_2O_2	-	Mn, Zn, Fe, Mg and Ca	F AAS	60	-	91–108%		(Dos Santos et al., 2019a)

Notes: [a]Ultrasound assisted-hydrogen peroxide extraction, [b]Ultrasound assisted-acid extraction, [c]inductively coupled plasma-optical emission spectroscopy, [c]flame atomic absorption spectroscopy, [d]Electrothermal atomic absorption spectroscopy, [e] Microwave-induced plasma optical emission spectrometry

4.3.6 Application of the validated methods in real pet food samples

The real pet food samples were subjected to the optimum conditions of both newly developed methods: MW-AHPD (microwave temperature: 200°C, digestion time: 40 mins, sample mass: 0.5 g, and H_2O_2 concentration: 5 mol/L) and UA-HPE (sonication time: 60 mins, sonication temperature: 80, sample mass: 0.1g, H_2O_2 concentration: 5mol). The resulted digests and extracts were analysed for the metal ions of interests (Cd, Pb, As, Cr and Sn) and the concentration results are shown in **Table 4.10 and Table 4.11**. As shown in both tables, the concentration levels of the toxic metals found in the pet food samples were compared to South African Fertilizers, Farm Feeds, Seeds, and Remedies Act 36 of 1947 and Food and Drug Administration (FDA) maximum tolerable levels. The levels of the investigated toxic metals ranged between 11.2-22.6, 6.4-11.9, 3.44-13.4, 0.44-2.98 and 0.18-0.98 µg/g for Cd, Pb, As, Cr and Sn, respectively for the MW-AHPD method. In the case of the UA-HPE method the investigated values of toxic metals ranged from 0.86-11.34, 4.50-11.45, 2.61-12.5, <DL-7.94, and <DL-1.04 µg/g for Cd, Pb, As, Cr, and Sn respectively.

In both methods the Fish (FBA and FBB) and wheat (WBA and WBB) pet food samples showed concentration levels above FDA and ACT 37/1947 limits for Cd, Pb, and As. The high concentration levels of As, Pb and Cd in fish and wheat samples might be due to use of contaminated fish and wheat-based ingredients as these are known to be susceptible to heavy metal contamination. The rest of the food ingredient groups (maize, brown rice, and leafy green vegetables); demonstrated desirable limits for all the toxic metals as per Act 36 of 1947 maximum tolerable limits. Moreover, different brands vary in concentration levels of the same food ingredients. A recent study on commercial pet food (Zafalon et al., 2021) found high levels of toxic metals in both cat and dog food, with Pb among those that exceeded the tolerable limits (9.13 ± 5.42 µg/g) in pet food, confirming the possibility of heavy metal contamination of pet food. Another study also confirmed the possibility of pet food contamination with heavy metals where cat food, on average contained higher concentrations of Cd (0.20 ± 0.01 µg/g), Cr (5.44 ± 1.42 µg/g), Pb (3.23 ± 0.08 µg/g), and Sn (299 ± 71µg/g) compared to dog food. Only Sn concentrated exceeded the tolerable limit (200 µg/g)(Abd-Elhakim et al., 2016). In another study conducted on dry dog food as well, fish-based diets were also found to contain higher amounts of cadmium, arsenic and mercury compared to poultry or red meat-based diet (Kim et al., 2018). The above-mentioned reports obtained similar results to this study, thus confirming the possibility of contamination of pet food with different heavy metals. Based on our current study analysis various pet food manufacturing companies might be using different strategies in processing, packaging, storing, and transporting pet food, which result in different food chemical qualities. Moreover, the pet food that showed concentration levels above prescribed limits by the standard bodies is a threat to the pet life, which might cause death. Therefore, the government must consider their compliance framework towards suppliers. Additionally, distributors of pet food such as supermarkets must request proof of analysis or certificates that certify that the products are of high quality and are safe.

Table 4.10: Concentration levels of metal ions expressed as µg/g in real pet food samples after digesting using MW-AHPD and ICP-OES analysis: Experimental conditions (sample amount, 0.5g; H_2O_2 concentration, 5 mol/L; microwave temperature, 200 °C, digestion time, 40 min and n=3)

Metal	**FBA**	**FBB**	**WBA**	**WBB**	**MBA**	**MBB**	**BRBA**	**BRBB**	**LGVBA**	**LGVBB**	**NRC[b]/FDA[c]**	**ACT 36/1947[d]**
Cd	**11.2±0.1**	**10.3±0.5**	3.6±0.2	9.41±0.06	8.32±0.79	6.41±0.01	2.6±1.3	1.49±0.09	2.34±0.4	4.12±0.01	10.0	10
Pb	**11.9±0.1**	**10.9±0.02**	6.49±0.03	6.93±0.7	8.75±0.1	8.32±0.04	6.70±0.09	7.94±0.06	7.40±0.2	6.9±0.4	10.0	10
As	**13.4±0.2**	8.10±0.1	**12.6±0.4**	**12.3±0.02**	6.01±0.5	9.02±0.2	4.11±0.09	6.17±0.3	3.44±0.09	4.77±0.03	12.5	12.5
Cr	2.30±0.7	0.44±0.1	1.94±0.6	2.58±0.5	1.32±0.01	0.44±0.3	<DL	2.98±0.7	1.09±0.7	2.03±0.4	10.0	-
Sn	0.98±0.3	0.42±0.01	<DL[a]	0.45±0.01	<DL	0.85±0.06	0.18±0.8	0.24±0.09	<DL[a]	<DL[a]	200	-

Notes: [a]Below detection limits, [b]National Research Council, [c]U.S. Food and Drug Administration, [d]Fertilizers, Farm Feeds, Seeds and Remedies Act 36 of 1947(South Africa).

Table 4. 11: Concentration levels of metal ions expressed as µg/g in real pet food samples after digesting using UA-HPE and ICP-OES analysis: Experimental conditions (sample amount, 0.1g; H_2O_2 concentration, 5 mol/L; sonication temperature, 80 °C, sonication time, 40 min and n=3)

Metal	**FBA**	**FBB**	**WBA**	**WBB**	**MBA**	**MBB**	**BRBA**	**BRBB**	**LGVBA**	**LGVBB**	**NRC[b]/FDA[c]**	**ACT 36/1947[d]**
Cd	**10.2±0.2**	**11.1±0.1**	4.7±0.4	**11.24±0.1**	7.33±0.37	5.76±0.2	4.17±0.9	0.96±0.1	1.85±0.3	5.01±0.45	10.0	10.0
Pb	9.21±0.2	**11.4±0.05**	5.74±0.01	4.44±0.1	7.94±0.3	7.28±0.09	5.74±0.01	8.21±0.02	8.01±0.6	5.74±0.1	10.0	10.0
As	**12.1±0.4**	9.24±0.5	**11.6±0.4**	**11.1±0.04**	5.98±0.1	9.77±0.13	5.43±0.11	5.94±0.14	2.91±0.3	4.09±0.01	12.5	12.5
Cr	7.84±0.1	0.98±0.54	2.32±0.5	3.07±0.1	2.14±0.20	<DL	<DL	2.42±0.24	2.07±0.1	1.91±0.25	10.0	-
Sn	0.84±0.2	<DL[a]	<DL[a]	0.33±0.21	<DL[a]	0.71±0.02	0.10±0.71	0.34±0.07	<DL[a]	<DL[a]	200	-

Notes: [a]Below detection limits, [b]National Research Council, [c]U.S. Food and Drug Administration, [d]Fertilizers, Farm Feeds, Seeds and Remedies Act 36 of 1947(South Africa).

4.3.7 AGREEPrep: an analytical greenness metric for the methods

AGREEPrep tool was used for assessment of the MW-AHPD and UA-HPE methods, and overall scores of 0.76, and 0.74 were obtained as shown in **Fig. 4.5**, demonstrating that the developed MW-AHPD and UA-HPE methods are green. The weak points for the both methods were criterion 1 (favour in situ sample preparation), criterion 7 (integrate steps and promote automation), and criterion 9 (chooses the greenest possible post -sample preparation configuration for analysis). Criteria 1 and 9 were below 0.2 and criterion 7 between 0.4 and 0.6. The rest of the criteria were strong, with 2, 4, 5, and 8 being the strongest points of this method and followed by criterion 3, 6, and 10.

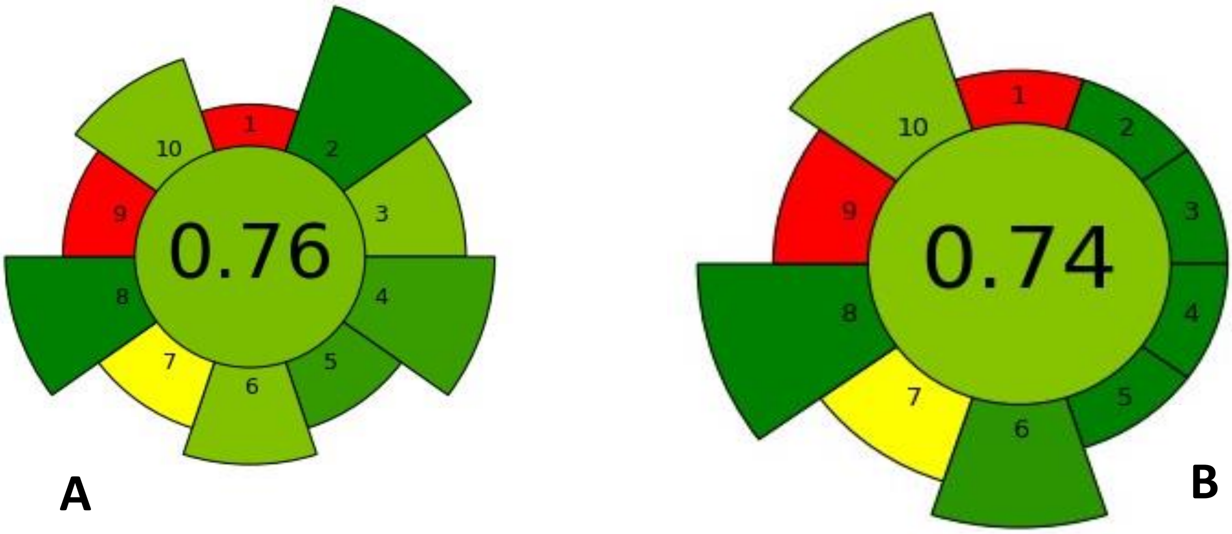

Figure 4. 5: AGREEPrep result of assessment and the corresponding color scale for assessment for this work (**A**: MW-AHPD, **B:** UA-HPE)

4.4 CONCLUSION

Green and rapid ultrasound-assisted hydrogen peroxide extraction (UA-AHPE) and microwave-assisted hydrogen peroxide digestion (MW-AHPD) methods were successfully optimised using multivariate experimental designs and validated using CRMs (fish muscle (ERMBB422 and wheat flour (FAP80467)). The newly developed MW-AHPD and UA-HPE methods were then applied for quantitative determination of As, Cd, Pb, Cr, and Sn in pet food samples by using inductively coupled plasma-optimal emission spectroscopy (ICP-OES). Furthermore, AGREEPrep metric tool was used for assessment of greenness of the MW-AHPD and UA-HPE. The overall scores of 0.76 and 0.74, respectively agreed that these newly develop methods are green. As a result, reduced ICP-OES background was observed in both methods as confirmed by the high sensitivity values which ranged between 4.2575 x 10^3 – 5.88 x 10^3 cps L mg^{-1} for Cd and 1.115 x10^3 3.2411 - 10^3 cps L mg^{-1} for As. The recovery results of the MW-AHPD and UA-HPE for the metals in the CRMs, demonstrated excellent accuracy of > 98%, and > 95%, respectively, for all the investigated metals, showing that the concentration levels obtained were in good agreement with the true value on the CRM certificate. For this reason, it can be concluded that proposed MW-AHD and UA-AHPE methods are accurate with minimal systematic errors. Furthermore, the obtained precisions for MW-AHPD and UA-HPE was below < 2.1 % and <1.9%, respectively, implying excellent reproducibility of both methods. The overall performance of the MW-AHPD and UA-HPE procedures were above the well-established microwave -assisted digestion and ultrasound-assisted extraction (UAE) sample preparation methods reported in literature.

The comparison results of the levels of metal ions between the regulating bodies and the newly developed method showed that As, Pb and Cd were the only metal ions that exceeded the maximum tolerable limits (Cd (11.2-22.6 µg/g), Pb (11.45-11.9 µg/g) and As (12.5-13.4 µg/g)) in some fish and wheat-based pet food samples. Furthermore, these three heavy metals were dominating in pet food in this descending order: As (2.61-12.5 µg/g)> Pb (4.50-11.45µg/g)> Cd (0.86-11.34 µg/g). The high concentration levels of As, Pb and Cd in fish and wheat-based pet food samples might be due contamination of fish and wheat ingredients. Fish is prone to heavy metal bioaccumulation from food, water and sediments in water sources, and wheat plants can easily take up the heavy metals from the soil and water from the root. Furthermore, pet food samples may have been contaminated with these heavy metals during processing, packaging, storage, and transportation.

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CHAPTER V (OVERALL CONCLUSION & FUTURE RECOMMENDATIONS)

PREAMBLE

Chapter five presents a summary of results of the present study, comparison of the two sample preparation methods (MW-AHPD and UA-HPE) in terms of their analytical figures of merits, and their greenness assessment scores, the conclusion drawn from the results obtained as well as future recommendations.

5.1 OVERALL CONCLUSION

The new greener sample preparation methods (UA-HPE and MA-HPD) were developed for multi-element determination of Cd, Pb, As, Sn, and Cr in pet food samples prior to analysis with ICP-OES. Both methods proved to be in harmony with green analytical chemistry, not only are they cost effective, rapid, simple, safe, they are also environmentally friendly. They use a green reagent, i.e. a dilute hydrogen peroxide. During extraction or digestion, dilute hydrogen peroxide decomposes into water and oxygen at high temperatures; hence the use of this solvent does not only reduce generated waste and lower solvent use, but it also reduces the risk associated with handling corrosive concentrated acids, toxic residues and reduces amount of acid vapours in the laboratory.

Additionally, when both methods were further assessed for greenness, the MA-HPD had a higher score due to its higher number of sample throughput per hour, not only does UA-HPE has longer extraction time (60 mins), but also the instrument cannot accommodate a lot of samples at a time compared to the microwave.

Application of both methods in maize, rice, vegetable, fish, and wheat-based pet food samples showed that Cd, Pb, and As were the metals which were above the tolerable limits (Cd: 10 µg/g), Pb :10 µg/g, As: 12.5 µg/g) for some wheat and fish-based pet food samples. In the MA-HPD method, the range of concentration of Cd, Pb, and As obtained were 11.2-22.6, 6.4-11.9, and 3.44-13.4µg/g respectively. In the UA-HPE, the range of concentration of Cd, Pb, and As were 0.86-11.34, 4.50-11.45, 2.61-12.55, respectively. The detected high concentration levels of Cd, Pb, and As, shows that there is possible heavy metal contamination in South African pet food industry.

5.2 FUTURE RECOMENDATIONS

The reported concentration of the As, Cd, Pb, Sn, and Cr showed that there is a gap in South Africa pet industry in terms of monitoring safety of pet food, further stringent monitoring measures need to be implemented. Moreover, there is also a need to continuously develop cheaper, environmentally friendly sample preparation methods which comply with green analytical chemistry for monitoring heavy metals in pet food. Additionally, there is also a need to assess the health risk of intake of heavy metal-contaminated pet food. Risk assessment will give more information about level of toxicity regarding heavy metal intake in pet food.

Therefore, further studies are required to trace the possible cause for the contamination of pet food by heavy metal ions. Moreover, these results suggest that there is a gap in the regulation of maximum tolerable levels of toxic metals in South African pet food. Therefore, the government authorities must find ways to enforce constant monitoring of heavy metals in pet food. Manufacturing companies need to ensure food safety and good quality of the food they process, and the distributors must purchase from reputable and food safety certified manufacturers and should request proof of the food safety and quality check before they purchase any stock to prevent selling intoxicated pet foods