**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**ELECTROCHEMICAL MECHANISTIC STUDIES AND
ELECTROCHEMICAL DETECTION OF CAFFEINE AND
CAPSAICINOLIDS EXTRACTED INTO AN ORGANIC
SOLVENT**

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SCHOOL OF PHYSICAL & MATHEMATICAL SCIENCES
NANYANG TECHNOLOGICAL UNIVERSITY

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Electrochemical Detection of Caffeine and
Capsaicinoids Extracted into an Organic
Solvent**

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Table of Contents

Abstract.....	i
List of Publications.....	ii
List of Abbreviations	iii
Chapter 1 Introduction.....	1
1.1.1. Food and Metabolism.....	2
1.1.2. Cytochrome P450 Enzymes	2
1.1.3. Electrochemistry for Metabolism Studies	3
1.1.4. Quantification of Food Molecules	3
1.2. Caffeine.....	4
1.2.1. Introduction to Caffeine	4
1.2.2. Previous Electrochemical and Chemical Studies on Caffeine	5
1.2.3. Various Detection Methods of Caffeine.....	5
1.3. Capsaicinoids	7
1.3.1. Introduction to Capsaicinoids	7
1.3.2. Previous Electrochemical Studies on Capsaicin	10
1.3.3. Various Detection Methods of Capsaicinoids.....	10
1.4. Aim of Project.....	12
1.5. References	13
Chapter 2 Experimental Techniques.....	19
2.1. Cyclic Voltammetry	20
2.2. Square-Wave Voltammetry.....	22
2.3. Differential-Pulsed Voltammetry	23
2.4. Controlled Potential Electrolysis.....	24
2.5. Rotating Disk Electrode Voltammetry	25
2.6. Karl-Fischer Titration.....	26
2.7. Experimental Procedures	27
2.7.1. Chemicals and Solvents	27
2.7.2. Supporting Electrolyte	28
2.7.3. Instrumentation	28
2.7.4. Voltammetric Experiments	28
2.7.5. Controlled Potential Electrolysis.....	30
2.7.6. Measurement of Water Content	31
2.8. References	32

Chapter 3 Electrochemically Controlled One-Electron Oxidation Coupled to Consecutive Hydrogen Atom Transfer of Caffeine.....	33
3.1. Experimental Procedures	34
3.1.1. Chemicals	34
3.1.2. Chemical Oxidation.....	34
3.1.3. Digital Simulations.....	34
3.2. Results and Discussion	35
3.2.1. Voltammetry of CAF in MeCN.....	35
3.2.2. Controlled Potential Electrolysis	39
3.2.3. Chemical Oxidation.....	41
3.2.4. Digital Simulations.....	42
3.3. Conclusion.....	44
3.4. References	45
 Chapter 4 Solid-Phase Extraction - Voltammetric Coupled Detection of Caffeine in Acetonitrile.....	 47
4.1. Experimental Procedures	48
4.1.1. Chemicals	48
4.1.2. Preparation of Stock Solution and Beverages	48
4.1.3. Sample Preparation.....	50
4.1.4. Instrumentation.....	51
4.1.5. Voltammetric Experiments.....	51
4.2. Results and Discussion	52
4.2.1. SWV of CAF in MeCN and Calibration Plot	52
4.2.2. DPV and SWV of CAF in PBS and Calibration Plots.....	53
4.2.3. SWV of Pre-filtered Extracted Real Beverage Samples in MeCN.....	56
4.2.4. DPV and SWV of Real Beverage Samples in PBS	58
4.2.5. SPE Coupled to Voltammetry Compared with Direct Voltammetric Determination of CAF	62
4.3. Conclusion.....	64
4.4. References	65
 Chapter 5 Electrochemical Behaviour of Capsaicin in Acetonitrile.....	 67
5.1. Experimental Procedures	68
5.1.1. Chemicals	68
5.1.2. Instrumentation.....	68
5.1.3. Voltammetric Experiments.....	68
5.2. Results and Discussion	70

5.2.1. Voltammetry of CAPH in MeCN	70
5.2.2. Voltammetry of CAPH with Varying Scan Rates.....	73
5.2.3. Controlled Potential Electrolysis.....	75
5.2.4. Rotating Disk Electrode Voltammetry	82
5.2.5. Proposed Mechanism	84
5.3. Conclusion	86
5.4. References.....	87

Chapter 6 Solid-Phase Extraction -Voltammetric Coupled Detection of Capsaicinoids in Acetonitrile 89

6.1. Experimental Procedures	90
6.1.1. Chemicals.....	90
6.1.2. Preparation of Stock Solution and Samples	90
6.1.3. Sample Preparation	90
6.1.4. Instrumentation	92
6.1.5. Voltammetric Experiments	92
6.2. Results and Discussion.....	93
6.2.1. Voltammetry of CAPs in MeCN with Varying Water Content	93
6.2.2. SWV of CAPs in MeCN with Varying Water Content and Calibration Plot.....	94
6.2.3. Extraction of CAPs from Samples	96
6.2.4. SWV of Post-SPE Sample Solution in MeCN	97
6.3. Conclusion	103
6.4. References.....	104

Chapter 7 Conclusion 105

Chapter 8 Appendix..... 109

Appendix 8.1.....	110
Appendix 8.2.....	126
Appendix 8.3.....	135
Appendix 8.4.....	140

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Abstract

Electrochemistry of food molecules have previously been studied in order to fundamentally understand their redox behaviour, and to see if this relates to their *in vivo* properties and to the metabolites that are formed. By employing electroanalytical techniques, the quantification of molecules in food samples could be achieved. Numerous electrochemical studies have been conducted in aqueous solution, focusing on the development of electrodes for quantitative purposes. However, very few studies were performed in organic solvents, which have similar environment to which non water soluble food molecules undergo metabolic reactions. Therefore, in this thesis, the electrochemistry of caffeine and capsaicinoids were explored in acetonitrile. The use of acetonitrile can reduce the chemical reaction between water in the solvent and the intermediate, allowing their redox behaviour, the intermediates and products all to be studied. In addition, detection methods for caffeine and capsaicinoids in acetonitrile were developed based on their electrochemical behaviour. Detection in acetonitrile allows for solid-phase extraction which aids in the removal of water-soluble interferences in samples. Moreover, the detection process is simple and easy with no electrode modification and could be performed in small volume, reducing reagents used.

List of Publications

The results in this thesis were published in the following research articles.

Research Articles:

CHAPTER 3

K. K. Chan, R. Ganguly, Y. Li, R. D. Webster, *ChemElectroChem* **2014**, *1*, 1557-1562.

CHAPTER 4

K.K. Chan, R. D. Webster, *Electroanalysis* **2016**, *28*, 516-522.

List of Abbreviations

CAF	caffeine
CAPH	capsaicin
CAPs	capsaicinoids
CPE	controlled potential electrolysis
CV	cyclic voltammetry
direct-V	direct - voltammetric method
DPV	differential-pulsed voltammetry
ESI	electrospray ionization
Fc	ferrocene
GC	glassy carbon
HMQC	heteronuclear multiple quantum correlation
HPLC	high performance liquid chromatography
MeCN	acetonitrile
MS	mass spectrometry
NADPH	nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
PBS	phosphate buffer solution pH 7.2
RDE	rotating disk electrode
SHU	scoville heat unit
SPE	solid-phase extraction
SPE-V	solid-phase extraction - voltammetric method
SWV	square-wave voltammetry
TRPV1	transient receptor potential cation channel subfamily V member 1
UV-vis	ultraviolet-visible light

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Introduction

Food, although obviously essential to life, was long thought to be a just a mixture of carbohydrates, protein and fats, until the study of certain diseases such as Beriberi and scurvy found that the illnesses were caused by a deficiency in certain vital minerals and nutrients present in the food consumed.^[1] The replenishment of the deficient nutrients in the body resulted in the cure from the illness.^[2] Subsequently, since the early 20th Century, much greater emphasis has been paid to food nutrition and there is a greater interest in the study of the individual molecular components of food. This led to the discovery of a series of vitamins which cannot be produced by our bodies. Other than vitamins, some herbs and spices are also known to possess medicinal properties.

Their extracts of some spices or herbs have long been used as treatments for certain illness or symptoms such as St. John's Wort for depression,^[3] eucalyptus for cough ^[4] and thyme for indigestion.^[5] The extracts are believed to contain an active principle, a compound responsible for the beneficial bioactivity. Isolation of the active principle has helped confirm the bioactivity of the extract. Some bioactivities observed in the active principle include antioxidant, anti-bacterial, analgesic and anti-inflammatory properties.

1.1.1. Food and Metabolism

Upon consumption, food molecules are broken down by a multitude of digestive enzymes before undergoing metabolism. Similar to drugs, food molecules also undergo Phase I and II metabolism. Phase I reactions result in the increase in polarity of the molecules to assist in the excretion from the body. They include the introduction of polar functional groups such as a hydroxyl group to non-polar molecules. Cytochrome P450 enzymes are important enzymes that are mostly involved in Phase I reactions. Phase II reactions result in further increase in polarity of the molecules formed from Phase I reactions which cannot be eliminated from the body or if reactive metabolites are generated from the reactions. They include the conjugation of hydrophilic groups or molecules such as glucuronate,^[6] acetate ^[7] and sulfate.^[8] Phase II reactions also tends to lead to the molecules being excreted in the urine.

1.1.2. Cytochrome P450 Enzymes

Cytochrome P450 enzymes are present in plants, bacteria and mammals and are highly expressed in the liver, gastrointestinal tract, lungs and kidney.^[9] While they have mixed functions, they are mostly responsible for the oxidation reactions. They are monooxygenase, so they catalyse the cleavage of dioxygen and incorporate one oxygen atom into the substrate while the other oxygen atom is reduced to water by two electrons. The two electrons are provided by nicotinamide adenine dinucleotide phosphate (NADPH) for this process. The overall reaction equation is as shown below in equation (1.1).

1.1.3. Electrochemistry for Metabolism Studies

The use of electrochemistry to study metabolic oxidation of food and drug molecules can be another useful tool to assist the current metabolism studies as the conditions are controlled and can simulate the conditions in biological systems. Furthermore, electrochemistry is cost effective and easy to perform. It can be particularly useful in examining toxicity of drugs before they go into further trials. Generating metabolites via electrochemical means have also been successful^[10] and it was found to match up quite well with that found *in vitro* and *in vivo*.^[11] Coupling electrochemistry with liquid chromatography-mass spectrometry was helpful in identifying reactive intermediates formed after the substrate is subjected to oxidation.^[10] Using various voltammetric techniques, a better idea of the bioactivity and chemical reactions that food molecules undergo can be obtained from their redox behaviour.

1.1.4. Quantification of Food Molecules

Quantification of food molecules traditionally require the extraction of the molecules, followed by identification by spectroscopic techniques before quantification is possible. With the technological advancement, more accurate instruments such as high performance liquid chromatography (HPLC) and liquid chromatography-mass spectroscopy are developed which can allow quantification of compounds present in smaller amounts. However, such machines are bulky, expensive, requires skilled operators to operate and might require complex sample pretreatment. Quantification through electroanalytical methods is more cost effective, easier to operate and can provide good sensitivity.

In this thesis, caffeine and capsaicinoids which are present in food and beverages widely consumed around the world will be the main focus. An introduction on the two molecules, electrochemical and chemical studies as well as detection methods performed on them up until now will be presented in the following sections.

1.2. Caffeine

1.2.1. Introduction to Caffeine

Caffeine (CAF), also known as 1,3,7-trimethylxanthine or 1,3,7-trimethyl-1H-purine-2,6(3H,7H)-ione, as shown in Figure 1.1, belongs to the purine family. It is a naturally-occurring alkaloid present in several plants such as coffee beans, tea leaves, cola nut as well as guarana seed. Although it was isolated in 1819 by Friedlieb Ferdinand Runge,^[12] caffeinated beverages had long since been consumed to reduce fatigue and help to keep one awake when feeling tired.^[13] The water solubility and liposolubility of CAF allows it to readily pass through the blood-brain barrier, while its structural similarity to adenosine causes it to act as an antagonist to the adenosine receptor, resulting in the perceived stimulating properties.^[14]

Figure 1.1: Molecular structure of caffeine.

Some adverse effects associated with caffeine consumption include anxiety, insomnia and nausea, and prolonged consumption of caffeinated beverages can result in addiction.^[15] Abstinence following prolonged consumption can give rise to withdrawal symptoms such as headaches, fatigue and lethargy.^[16] Despite the undesirable effects associated with prolonged consumption at high concentrations, it was found that CAF could act as an antioxidant with concentration dependent activity.^[17] CAF was recently reported to be an effective scavenger for hydroxyl (HO[•]) radicals with a bimolecular rate constant k of $2.6 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$.^[18] Furthermore, the antioxidant

properties of CAF have also been proposed to be protective against Alzheimer's disease^[19] and the formation of cataracts caused by reactive oxygen species.^[20]

1.2.2. Previous Electrochemical and Chemical Studies on Caffeine

An electrochemical study on CAF was first performed in an aqueous medium by Hansen and Dryhurst to simulate the biological oxidation of CAF in the body.^[21] The oxidation process was found to be chemically irreversible and was proposed to involve four electrons and four protons. An initial two electrons and two protons oxidation of the C8-N9 bond yielded 1,3,7-trimethyluric acid, a metabolite of CAF, followed by another two electrons and two protons oxidation of the C4-C5 bond to give a 4,5-diol intermediate, which could undergo further reaction. *In-situ* generated radicals such as persulfate ($\text{SO}_4^{\bullet-}$),^[22] hydroxyl ($\text{OH}^{\bullet}$)^[22] and phosphate ($\text{PO}_4^{2\bullet-}$)^[23] radicals were employed to examine the oxidation mechanism in aqueous solutions. It was verified by HPLC that 1,3,7-trimethyluric acid was the main product formed between CAF and $\text{SO}_4^{\bullet-}$, $\text{OH}^{\bullet}$ and $\text{PO}_4^{2\bullet-}$. It was suggested that the formation of 1,3,7-trimethyluric acid by the reaction between CAF and $\text{SO}_4^{\bullet-}$ and $\text{PO}_4^{2\bullet-}$ was due to the reaction of the CAF radical cation ($\text{CAF}^{\bullet+}$) with water followed by deprotonation and further oxidation.

1.2.3. Various Detection Methods of Caffeine

Various methods have been developed to determine the CAF content in beverages, including high-performance liquid chromatography (HPLC),^[24] thin layer chromatography,^[25] gas chromatography,^[26] ultraviolet-visible light spectroscopy,^[27] capillary electrophoresis,^[28] immunoassay^[29] and fluorescence spectroscopy.^[30] However, most of these methods require complex sample pretreatment or expensive instruments which require trained personnel to operate. Attempts at electrochemical detection using various carbonaceous electrodes such as a graphite pencil electrode,^[31] an edge plane pyrolytic graphite electrode,^[32] a boron-doped diamond electrode,^[33] and a carbon paste microelectrode^[34] were made. Modified carbon electrodes such

as a Nafion-coated electrode,^[35] a polymer modified electrode,^[36] and a multiwalled carbon nanotube and gold nanoparticle molecularly imprinted polymer electrode^[37] have been developed to achieve better selectivity and sensitivity for the detection of CAF.^[38] Despite the development of various carbonaceous electrodes for the determination of CAF, preparation of these electrodes can be tedious and time-consuming and the modified electrodes may not be entirely reproducible between measurements, especially if the preparation method does not lead to electrodes with identical surface areas.

1.3. Capsaicinoids

1.3.1. Introduction to Capsaicinoids

Capsaicin is known to be one of the most abundant capsaicinoids in hot chilli.^[39] Capsaicinoids are a class of compounds with structural similarity to capsaicin. They are recognised by the three sections, namely the vanillyl functional group on one end, the amide group in the middle and the aliphatic carbon tail on the other end as shown in Figure 1.2. Due to the presence of the hydrogen atom in the hydroxyl functional group, capsaicin will be abbreviated CAPH to facilitate explanation later in Chapter 5.

Capsaicinoids are usually differentiated by the differing degrees of unsaturation and length of their aliphatic carbon chain. There are currently thirteen known capsaicinoids which are presented in Table 1.1.^[39-40] Thus, capsaicinoids will be abbreviated CAPs to encompass all possible capsaicinoids and prevent confusion with capsaicin (CAPH). CAPs are known to elicit a spicy or "hot" sensation when consumed, inhaled or when in contact with the skin, due to the activation of the transient receptor potential cation channel subfamily V member 1 (TRPV1) receptor in sensory neurons.^[41] Due to the uncomfortable experience upon consumption, they were found to be useful as deterrents or repellents to mammals and pests.^[42] Humans, on the other hand, have adapted to its spiciness and chilli is now widely consumed around the world and is used mostly as food seasoning and flavouring. Besides being used in food, it is known to be analgesic^[43] and has been incorporated in gels, creams and plasters for pain-relief. It has also been reported to exhibit antimicrobial properties,^[44] anti-inflammatory properties,^[45] anti-tumoral properties,^[46] anti-mutagenic properties,^[47] and antioxidant properties.^[48] Understanding the metabolic pathway that capsaicinoids undergo might aid in understanding their beneficial bioactivities.

Figure 1.2: Molecular structure of capsaicin (CAPH).

Table 1.1: Capsaicinoids and their respective aliphatic carbon chain group (R).

Capsaicinoids	Aliphatic group (R)
Capsaicin
Dihydrocapsaicin
Nordihydrocapsaicin
Norcapsaicin
Homocapsaicin I
Homocapsaicin II
Homodihydrocapsaicin I
Homodihydrocapsaicin II
Nornorcapsaicin
Nornordihydrocapsaicin
Octanamide
Nonivamide
Decanamide

1.3.2. Previous Electrochemical Studies on Capsaicin

The first electrochemical study on capsaicin was performed by Boersch et al.^[49] Fouling of the electrode after electrochemical oxidation was observed. The electrochemical oxidation mechanism was later studied in detailed by Kachosangi et al. in an acidic aqueous solution.^[50] Capsaicin was noted to adsorb onto the electrode surface after electrochemical oxidation. The oxidation process involved two-electron and one proton followed by an irreversible chemical step to form an *o*-quinone functional group on capsaicin. The *o*-quinone moiety then underwent a reversible redox process to form a catechol functional group on reduction and regenerate back the *o*-quinone functional group on oxidation. Various electrochemical studies on CAPH in aqueous solution also verified this observation.^[51] The key intermediate involved in the oxidation process was proposed to be the phenoxonium cation, which formed the *o*-quinone functional group upon hydrolysis of the 2-methoxy group with the aid of water in the solvent medium.

1.3.3. Various Detection Methods of Capsaicinoids

One of the earliest method of quantifying capsaicinoids (CAPs) was the Scoville Organoleptic Test invented by Wilbur Scoville in 1912, and it quantified CAPs by Scoville Heat Units (SHU).^[52] In the method devised by Scoville, CAPs from an exact weight of dried chilli, were extracted into alcohol and subsequently diluted with sugar water. The diluted extract is given to a panel of professional tasters for tasting. The extract would be further diluted until a majority of the tasters do not feel the heat in the extract. The heat level is decided based on the dilution factor. While this method is simple, the results are very subjective as the heat level is dependent on the response of the professional tasters and the tasters might be desensitised by the CAPs after a few samples.

Other methods of quantifying CAPs have thus been developed including colorimetric determination,^[53] thin layer chromatography,^[54] gas chromatography-mass spectrometry,^[40c, 55] HPLC,^[40c, 40g, 48a, 56] and liquid chromatography-mass spectrometry.^[40c, 40f, 56c, 57] Most of the

instruments used are expensive, with complex sample pretreatment that require skilled personnel to operate. Electrochemical methods, on the other hand, are inexpensive, easy to operate and can provide good sensitivity, but often suffer from more interferences compared to the chromatographic methods.

Kachoosangi et al. reported that in acidic aqueous solution CAPH adsorbed onto the working electrode after oxidation and thus, adsorptive stripping voltammetry was employed using a multiwalled carbon nanotube screen-printed electrode for quantification.^[50] Many electrochemical methods using various electrodes for the detection of CAPs were later developed, such as a boron doped diamond electrode,^[51a] an amino-functionalised mesoporous silica modified carbon paste electrode,^[51b] a ferrocene mediated horseradish peroxidase enzyme modified screen printed carbon paste electrode,^[58] a pencil graphite electrode,^[51e] a gold nanoparticle decorated multiwalled carbon nanotube modified glassy carbon electrode,^[59] a back-to-back single-walled carbon nanotube screen printed electrode,^[51f] a mesoporous cellular foam modified carbon paste electrode,^[51g] microparticles immobilised on paraffin-impregnated graphite electrode,^[60] graphene-titania-Nafion composite film modified glassy carbon electrode,^[51h] phenylalanine ammonia-lyase enzyme immobilised using Nafion on multiwalled carbon nanotube modified platinum electrode^[61] and an Ag/Ag₂O nanoparticles/reduced graphene oxide modified screen-printed electrode.^[51i]

1.4. Aim of Project

In this thesis, to achieve a better understanding of the intermediates as well as products formed during oxidation, electrochemical studies of caffeine and capsaicin were conducted in the aprotic organic solvent, acetonitrile. Acetonitrile, as a solvent, is known to stabilise intermediates.^[62] Since these two food molecules are liposoluble, the use of an organic solvent can also provide an environment similar to that experienced by the molecules in the body where oxidation reactions can occur. The study of the redox behaviour of the molecules under low and high water content is also made possible with the use of an organic solvent. The number of electron transferred for each voltammetric process as well as the formation of long-term products were studied with controlled potential electrolysis. Variation in scan rate during cyclic voltammetric (CV) measurements helps to provide an idea on the lifetime of the intermediates.

Based on the electrochemical behaviour found, an electrochemical quantification method involving the detection of the two food molecules in acetonitrile was developed. Extraction of the two food molecules from the samples into acetonitrile prior to detection was required to reduce water-soluble interferences present in the samples and to change the sample medium into one suitable for voltammetric measurement. The detection was done on a bare electrode which did not require any preparation. The results obtained voltammetrically were compared with that of HPLC.

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Experimental Techniques

Various techniques were applied throughout the studies in this thesis. Electrochemical techniques such as cyclic voltammetry, square-wave voltammetry, differential-pulsed voltammetry, controlled-potential electrolysis, rotating disk electrode voltammetry were used for electrochemical studies. The water content of dried solutions were obtained using a Karl Fischer coulometric titrator.

2.1. Cyclic Voltammetry

Cyclic Voltammetry (CV) is an electrochemical technique which is useful to understand the electrochemical behaviour of an analyte following the transfer of an electron or multiple electrons. In a typical experiment, the working electrode potential applied with respect to the reference electrode is allowed to scan from a potential E_0 to E_1 , the switching potential. The scan direction is then reversed and the potential is scanned back to E_0 in a linear fashion against time in a saw tooth waveform as shown in Figure 2.1. This cycle can be repeated as many times as needed. For oxidation processes, the potential is scanned in the positive potential direction, which results in positive anodic current observed. Conversely, the potential is scanned in the negative potential direction for reduction processes and negative cathodic current will be observed. The rate at which the potential varies with respect to time is known as scan rate, ν (V s^{-1}).

The current recorded between the working electrode and the counter electrode during the cycle is plotted against the potential applied, which gives a cyclic voltammogram as shown in Figure 2.1. The cyclic voltammogram as shown in Figure 2.1 is typical of an analyte **A** with a reversible redox couple. As the time increases from t_0 to t_1 , an increasing potential from E_0 to E_1 is applied. The concentration of **A** at the surface of the electrode decreases, resulting in a concentration gradient towards the electrode. Thus, the anodic current would increase with time. At a certain point in time, despite increasing applied potential, the consumption of **A** at the electrode surface cannot be replenished by the diffusion of **A** to the electrode surface and so the current is limited by the diffusion of **A** towards the electrode. At further potentials, the current decays with the concentration of the analyte at the electrode surface. During the reverse scan from time t_1 to t_2 , the oxidised A^{++} can be reduced, a current of the reverse polarity (cathodic current) would increase as the amount of A^{++} formed at the electrode surface gets reduced back to **A**. After which, the current would decay back to zero. If the heterogeneous electron transfer is fast and the electrochemical behaviour is reversible, the peak current i_p , limited by diffusion of the analyte to the electrode surface, could be described by Randles-Sevcik Equation as shown below in equation (2.1).

$$i_p = 0.4463 n F A C \left(\frac{n F v D}{R T} \right)^{1/2} \quad \text{equation (2.1)}$$

where i_p is the peak current (A), n is the number of electrons transferred, F is the Faraday constant (96,485 C mol⁻¹), A is the area of the electrode (cm²), C is the concentration of the analyte (mol cm⁻³), v is the scan rate (V s⁻¹), D is the diffusion coefficient of the analyte (cm² s⁻¹), R is the gas constant (8.314 J K⁻¹ mol⁻¹) and T is the temperature (K).

With the assumption that the temperature is 298 K, equation (2.1) could be simplified to obtain equation (2.2) below.

$$i_p = 268600 n^{3/2} A D^{1/2} C v^{1/2} \quad \text{equation (2.2)}$$

where i_p is the peak current (A), n is the number of electrons transferred, A is the area of the electrode (cm²), D is the diffusion coefficient of the analyte (cm² s⁻¹), C is the concentration of the analyte (mol cm⁻³) and v is the scan rate (V s⁻¹).

Figure 2.1: (Left) The potential profile in the form of a saw tooth waveform of potential E as a function of time t . (Right) Cyclic voltammogram of analyte A in a plot of current i as a function of potential applied E .

A proportional plot of the anodic peak current against the square root of scan rate is characteristic of a diffusion-controlled electrochemical process. One that deviates from such proportionality would usually be associated with an adsorption-controlled electrochemical process.

An analyte which produces an anodic peak current (i_p^{ox}) and cathodic peak current (i_p^{red}) with similar size ($i_p^{red} / i_p^{ox} \approx 1$) can be considered to be chemically reversible and the peak-to-peak separation of the anodic and cathodic peaks would be $57/n$ mV for an analyte with n e⁻ process. When (i_p^{red} / i_p^{ox}) is less than or more than one, the electrochemical behaviour of the analyte may not be chemically reversible.

2.2. Square-Wave Voltammetry

Square-Wave Voltammetry (SWV) is a rapid electrochemical differential technique that has a superposition of a staircase waveform and a step cycle waveform ^[1] as shown in Figure 2.2, developed by Osteryoung et. al.^[2] The current is sampled twice and the difference in current (Δi) is plotted against the potential applied. As the non-faradaic current due to the charging of double layer is high after the initial pulse and decays faster than the faradaic current of the analyte, current is sampled before the end of the first half cycle (forward cycle) and at the end of the first cycle (reverse cycle) as displayed in Figure 2.2, allowing only the sampling of faradaic current. The difference in current further eliminates residual non-faradaic current. At potentials before the redox potential of the analyte, the forward current and reverse current are zero. At potentials past the redox potential, the currents are diffusion controlled. Hence, the forward and reverse currents should cancel out, resulting in a symmetrical peak. Thus, the difference in current would be largest at the redox potential. As current is directly proportional to concentration, the peak height (Δi) at the redox potential can be used for quantification purposes.

Figure 2.2: (Left) Potential profile of square-wave voltammetry (Solid line) in which a step cycle waveform (Dotted line) is superimposed onto a staircase waveform (Dashed line).^[1] Current is sampled at the end of the forward cycle (Black circle) and reverse cycle (White circle). (Right) The difference in current sampled at the forward cycle (Red line) and reverse cycle (Blue line), Δi , is plotted as a function of potential applied, E (Black line).

2.3. Differential-Pulsed Voltammetry

Differential-Pulsed Voltammetry (DPV) is an electrochemical differential technique similar to SWV. A fixed potential pulse of a small amplitude superimposed onto a staircase voltage.^[1] Current is sampled twice before the pulse and at the end of the pulse as shown in Figure 2.3 and the difference in current (Δi) is plotted against the base potential.

Figure 2.3: (Left) Potential profile of differential-pulsed voltammetry in which a fixed potential pulse applied at intervals (Dotted line) is superimposed onto a staircase waveform (Dashed line).^[1] Current is sampled before the pulse (White circle) and at the end of the pulse (Black circle). (Right) The difference in current sampled at the end of the pulse (Red line) and before the pulse (Blue line), Δi , is plotted as a function of potential applied, E (Black line).

2.4. Controlled Potential Electrolysis

Controlled Potential Electrolysis (CPE) is used mainly to determine the number of electrons transferred during the redox reaction as well as to scale up the amount of the long term products formed after oxidation or reduction. A working electrode of a large surface area such as a mesh or cylinder is often used during CPE to speed up the electrolysis process. Bubbling of the test solution with inert gas such as nitrogen or argon during the CPE assists in deoxygenating the solution as well as facilitating the transfer of the electrolysed product away from the working electrode and allowing more analyte to undergo CPE.

CV is usually performed prior to CPE to locate the anodic or cathodic peak potential of the analyte. A potential of 100 to 200 mV more positive (for oxidation) or negative (for reduction) than the peak potential is applied during CPE to ensure that the analyte can be electrolysed exhaustively. A plot of current against time is recorded as CPE progresses. The current is allowed to decay until a negligible current level is observed before the CPE is stopped. The integration of

current recorded with respect to the time passed gives the total number of electrons transferred.^[3]

As the mass of the analyte used in CPE is known, the number of electrons, n for the electrochemical process studied can be obtained from equation (2.3) shown below.^[4]

$$n = Q / N F \quad \text{equation (2.3)}$$

where Q is charge in coulombs (C), N is the number of moles of the starting material and F is the Faraday constant (96,485 C mol⁻¹).

2.5. Rotating Disk Electrode Voltammetry

Linear sweep voltammetry is usually performed in conjunction with a rotating disk electrode (RDE) or a microelectrode. The potential is applied with respect to the reference electrode linearly from one potential to another potential. As the applied potential is scanned closed to the redox potential of the analyte **A**, the current increases as the concentration of **A** at the electrode surface decreases, resulting in a concentration gradient towards the electrode surface. Due to the rotation of the electrode, a constant flux of analyte is provided to the electrode surface. Thus, once the applied potential is past the redox potential, a constant limiting current i_L not controlled by the diffusion of the analyte to the electrode but by the rotational speed of the electrode is as depicted by Levich equation in equation (2.4) as shown below. Thus, RDE voltammogram typically gives a sigmoidal curve.

$$i_L = 0.620 n F A D^{2/3} \omega^{1/2} \nu^{-1/6} C \quad \text{equation (2.4)}$$

where i_L is the peak current (A), n is the number of electrons transferred, F is Faraday constant (96,485 C mol⁻¹), A is the area of the electrode (cm²), D is the diffusion coefficient (cm² s⁻¹), ω is the rotational speed (rad s⁻¹), ν is the kinematic viscosity (cm² s⁻¹) and C is the concentration of the analyte.

The RDE voltammetric experiment is useful for the determination of diffusion coefficients or the number of electrons transferred. From the gradient of a linear plot of the limiting current (i_L) with

the square root of rotational speed ($\omega^{1/2}$), mathematical manipulation with the knowledge of several other variables allows the values of n or D to be obtained.

2.6. Karl-Fischer Titration

Karl-Fischer titration, first invented by Karl Fischer in 1935, can be used to measure the trace water content present in a solution via coulometric or volumetric titration.^[5]

The titration cell is usually comprised of two compartments, an anodic compartment (the sample solution is injected into) and a cathodic compartment. The anodic solution is obtained as an anolyte which is made up of an alcoholic solvent (methanol), sulphur dioxide, iodide and a base (imidazole). The cathodic compartment is immersed in the anolyte separated by a membrane.

Karl-Fischer coulometric titration is based on the chemical reactions as shown below in the following equations (2.4)-(2.6), where B is the base and ROH is the alcoholic solvent.

I_2 is produced electrochemically at the Pt anode in the anolyte solution, while hydrogen is generated at cathode. Based on the stoichiometric ratio of I_2 and H_2O in equation (2.6), one mole of H_2O consumes one mole of I_2 . During the titration process, a constant current is maintained between the two electrodes to generate I_2 for the reaction with H_2O . When the titration process is completed, there would be excess I_2 in the anolyte so a drop in potential will be detected between a pair of indicator electrodes. The amount of charge required to convert I^- to I_2 with the knowledge of the mass of the sample injected can thus be used to calculate the amount of water content present in the sample solution.

2.7. Experimental Procedures

The experimental procedures mentioned in this section are generic, detailed procedures pertaining to particular experiments are further described in the individual chapters.

Unless otherwise specified, all chemicals and reagents were purchased from commercial sources and used as received. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance 500 (AV 500) spectrometer equipped with an Ultrashield Plus 500 MHz magnet with BBI probe (5 mm). HMQC NMR spectra were recorded on a Bruker Avance III 400 (BBFO 400) spectrometer equipped with an Ultrashield Plus 400 MHz magnet with auto-tunable BBFO probe (5 mm). Chemical shifts (parts per million) were recorded with tetramethylsilane (TMS) as the internal reference standard. Multiplicities are given as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublets). The number of protons (n) for a given resonance is indicated by $n\text{H}$ and coupling constants are reported as a J value in Hertz (Hz).

2.7.1. Chemicals and Solvents

Caffeine (pure Ph. Eur., USP) was obtained from AppliChem. Capsaicin (natural grade, > 60 %) also called 8-methyl-*N*-vanillyl-trans-6-nonenamide (CAPH) and nonivamide (> 96 %) also named *N*-vanillylnonanamide were purchased from TCI. Pyrocatechol was purchased from Merck. Vanillin (99 %) was purchased from Alfa Aesar. DL- α -tocopherol acetate was purchased from Sigma Aldrich. Acetonitrile, ethanol and methanol used in all experiments were either high-performance liquid chromatography or analytical reagent grades. Molecular sieves, in the form of $1/16$ in. rods with 3 Å pore size (CAS 308080-99-1) were obtained from Fluka, and were preheated under vacuum at 433 K for 6 hours followed by cooling before use. Purified water, with a resistivity of $\geq 18 \text{ M}\Omega\text{cm}$, was obtained from an ELGA Purelab Option-Q system.

2.7.2. Supporting Electrolyte

The supporting electrolyte used in voltammetric experiments in MeCN, tetra-*n*-butylammonium hexafluorophosphate (*n*-Bu₄NPF₆), was prepared following a literature procedure^[6] by reacting equimolar amounts of aqueous solutions of tetra-*n*-butylammonium hydroxide (*n*-Bu₄NOH, 40 %, Alfa Aesar) and hexafluorophosphoric acid (HPF₆, 65%, Fluka), washing the white precipitate with water, recrystallizing three times from hot ethanol and drying under vacuum at 413 K for 6 h and storing in a desiccator until use.

2.7.3. Instrumentation

All voltammetric experiments were conducted on a computer controlled Eco Chemie Autolab PGSTAT302N potentiostat controlled by GPES or NOVA software. High performance liquid chromatography (HPLC) analysis was performed using Agilent 1200 Series (Model G1311A) system with a UV-vis detector (Model G1365B) set at 268 nm. ZORBAX Eclipse XDB-C18, 5 μ m, 150 mm $\times$ 4.6 mm column was used.

2.7.4. Voltammetric Experiments

Voltammetric experiments were performed in a three-electrode electrochemical cell. A 1 mm diameter planar platinum (Pt) and/or glassy carbon (GC) disk working electrodes (eDAQ Pty Ltd) and Pt wire auxiliary electrode (Metrohm) were used for CV experiments. A miniature silver (Ag) wire reference electrode (eDAQ Pty Ltd) used for voltammetric experiments in MeCN was connected to the test solution via a salt bridge containing 0.5 M *n*-Bu₄NPF₆ in MeCN. All voltammetric experiments were performed in a Faraday cage at 295 ($\pm$ 2) K and under an argon atmosphere. Before each scan, the working electrode was polished with alumina oxide (grain size 0.3 μ m) slurry on a Buehler Ultra-pad polishing cloth, rinsed with acetone and dried and the test solution was deoxygenated by purging with argon gas. All potential values for CV experiments were reported with reference to ferrocene (Fc) as an internal standard, which was added to the test

solution at the end of measurements. A typical set up for voltammetric experiments is as shown in Figure 2.4.

Figure 2.4: Set up for voltammetric experiment in electrochemical cell.

The dried solution containing supporting electrolyte was prepared by adding preheated molecular sieves into the solution which was kept in a vacuum syringe (SGE Analytical Science) for at least one day prior to use. For voltammetric experiments involving dried conditions, the electrochemical cell was heated at 373 K for two hours and allowed to cool to room temperature under an argon atmosphere. The dried solution was then injected into the cell and the solution was deoxygenated by purging with argon gas.^[7]

2.7.5. Controlled Potential Electrolysis

CPE experiments were performed in a two-compartment electrolysis cell separated by a porosity no. 5 (1.0–1.7 μm) sintered glass frit.^[8] Pt meshes or GC cylinders of identical size could be used as the working and auxiliary electrode. A miniature Ag wire reference electrode used in the experiments, connected to the test solution via a salt bridge containing 0.5 M $n\text{-Bu}_4\text{NPF}_6$ in MeCN, was placed in the working electrode compartment. The volumes of the solution in the auxiliary and working electrode compartment were approximately 25 mL. The solutions in both working and auxiliary electrode compartment were purged with argon gas before the experiment to get rid of any dissolved oxygen in the solution. During CPE, the solutions were bubbled to encourage stirring and to provide an inert atmosphere. All potentials for CPE experiments were obtained and reported with reference to the Ag wire reference electrode or ferrocene (Fc) as stated in the chapter. A typical set up for controlled potential electrolysis experiments is as shown in Figure 2.5.

Figure 2.5: Set up for controlled potential electrolysis experiment.

2.7.6. Measurement of Water Content

The water content was measured by performing Karl Fischer titrations with a Mettler Toledo DL32 coulometer using (Riedel-deHaën) HYDRANAL-Coulomat AG and HYDRANAL-Coulomat CG as the anolyte and catholyte, respectively. The coulometer was allowed to stabilise until a steady drift value close to $0 \mu\text{gmin}^{-1}$ of water was achieved before measurements were taken. Measurements were carried out under constant humidity (30 %) in a (122 cm $\times$ 61 cm $\times$ 61 cm) humidity control box using a dry nitrogen gas purge system from Coy Laboratory Products Inc.^[7] A 10 mL vacuum syringe (SGE Analytical Science) was used to draw approximately 3 mL aliquots of the electrochemical solution from the electrochemical cell. The water content was determined by doing three successive 1 mL solution injections into the coulometer and taking an average of the three measurements. The set up for the Karl-Fischer titrator is as shown in Figure 2.6.

Figure 2.6: A Karl-Fischer coulometric titrator.

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Electrochemically Controlled One-Electron Oxidation Coupled to Consecutive Hydrogen Atom Transfer of Caffeine

Previous electrochemical studies on the oxidation of caffeine (CAF) in aqueous solution reported it to be chemically irreversible and the intermediate formed was proposed to undergo a chemical reaction with water.^[1] In this chapter, the reaction mechanism of CAF oxidation was examined using electrochemical methods in the aprotic organic solvent acetonitrile (MeCN). It is thought that such a medium could help to stabilise reactive intermediates^[2] and provide an environment more closely related to the lipophilic environment where lipid-soluble CAF can also undergo metabolic oxidation reactions. Furthermore, any chemical reactions between intermediate formed and water could be reduced significantly in aprotic organic media, allowing a better characterization of the intermediate.

3.1. Experimental Procedures

3.1.1. Chemicals

Nitrosonium hexafluoroantimonate, (NOSbF₆, 99.9 %) used for chemical oxidation experiments was obtained from Sigma-Aldrich and stored in a glovebox under a nitrogen atmosphere. MeCN under undried conditions was used directly from the solvent bottle with a water content of approximately 70–80 mM. MeCN under dried conditions was achieved by adding preheated molecular sieves into the solution, giving a lower water content of approximately 10–25 mM (when placed in the electrochemical cell containing a reference electrode).^[3] Acetonitrile-d₃ (99.8 % D) for NMR experiments was bought from Cambridge Isotope Laboratories Inc. in ampoule form and was used directly from the ampoule.

3.1.2. Chemical Oxidation

CAF (9.38 mg) was dissolved in 1.0 mL of dried MeCN and then added to NOSbF₆ (12.97 mg). The solution was bubbled with argon following the addition to remove any gaseous NO by-product formed. Removal of solvent allowed the formation of a white solid. (20.45 mg, 98.2 %). m. p. 238 °C (decomp.); ¹H NMR (CD₃CN, 500 MHz) δ 3.31 (s, 3H, Me), 3.49 (s, 3H, Me), 4.09 (s, 3H, Me), 8.53 (s, 1H, CH); ¹³C {¹H} NMR (CD₃CN, 500 MHz) δ 29.22, 32.58, 37.21, 108.56, 137.00, 141.02, 151.40, 155.04.

3.1.3. Digital Simulations

Digital simulations of the CV data were performed using the DigiElch software package.^[4]

3.2. Results and Discussion

3.2.1. Voltammetry of CAF in MeCN

CAF can readily dissolve in many organic solvents suitable for electrochemistry, and MeCN was chosen because it provides a wide potential window and is relatively unreactive. Previous studies examining CAF in aqueous solutions favoured the use of a GC working electrode because it has a higher overpotential for hydronium ion reduction compared to Pt electrode.

In the preliminary experiments, the oxidation of 2 mM CAF in MeCN containing 0.2 M *n*-Bu₄NPF₆ was examined on both Pt and GC electrodes as they are electrochemically inert and have wide potential windows in MeCN. To determine the effect of water on the oxidation of CAF, CV on each electrode were performed under both undried and dried conditions (see Experimental Procedures). Under undried conditions, an anodic peak on the forward scan at ca. 1.2 V vs. Fc/Fc⁺ with no corresponding cathodic peak (but with two small cathodic peaks on the reverse scan at ca. 0 V and -0.5 V vs. Fc/Fc⁺) was observed when a Pt electrode was used as shown in the voltammogram in Figure 3.1 (Red solid line). When a GC electrode was used, three anodic peaks at ca. 1.2 V, 1.35 V and 1.7 V vs. Fc/Fc⁺ on the forward scan were detected but no cathodic peaks on the reverse scan were observed in Figure 3.1 (Blue solid line). Under dried conditions, two anodic peaks at ca. 1.2 V and 1.6 V vs. Fc/Fc⁺ with a corresponding cathodic peak at ca. 1.1 V vs. Fc/Fc⁺ were observed at a Pt electrode (Figure 3.1, Red dotted line). When a GC electrode was used under dried conditions, three anodic peaks at ca. 1.2 V, 1.5 V and 1.7 V vs. Fc/Fc⁺ on the forward scan and a corresponding peak at ca. 1.1 V vs. Fc/Fc⁺ on the reverse scan were observed (Figure 3.1, Blue dotted line).

Figure 3.1: CV of 2 mM CAF in MeCN containing 0.2 M $n\text{-Bu}_4\text{NPF}_6$ using 1 mm diameter Pt (Red line) and GC (Blue line) electrodes recorded at 0.1 V s^{-1} . Solid line (—) denotes undried conditions with high water content of ca. 70–80 mM and dotted line (.....) denotes dried conditions with lower water content of ca. 10–25 mM.

Based on the results shown in Figure 3.1, the lifetime of the radical cation formed after oxidation at ca. $1.2 \text{ V vs. Fc/Fc}^+$ was significantly lengthened under dried conditions compared to undried conditions, since the reverse cathodic peak at ca. $1.1 \text{ V vs. Fc/Fc}^+$ was much larger under dried conditions. This indicates that it is likely that the water present in undried MeCN is increasing the reactivity of the cation formed during electrochemical oxidation. As the cathodic peak corresponding to the reduction of the initially formed radical cation was larger on a Pt electrode compared to GC electrode (under the same conditions), the Pt electrode surface was chosen as the working electrode for further investigation of the oxidation of CAF in MeCN. In the following results, the potential window was limited to the first oxidation peak to prevent unwanted further oxidation of the initially formed cation.

To determine the lifetime of the cation produced during the first oxidation process, CV experiments at various scan rates between 0.1 Vs^{-1} and 20.0 Vs^{-1} were performed. Since the background current is more significant at faster scan rates, the CV data were background subtracted by recording variable scan rate CVs in the absence of CAF and digitally subtracting

them from the CVs obtained in solutions containing CAF. By varying the scan rates, the anodic (i_p^{ox}) to cathodic (i_p^{red}) peak current ratio (i_p^{ox}/i_p^{red}) for the oxidation process was observed to approach unity as the scan rate reaches approximately 10 Vs^{-1} as shown in Figure 3.2. A small cathodic peak at -0.4 V vs. Fc/Fc^+ was also observed on the reverse scan at slow scan rates only after first oxidising CAF, suggesting that this peak could be due to the reduction of an oxidised product.

Figure 3.2: Background-subtracted CV of 2 mM CAF in dried MeCN containing 0.2 M $n\text{-Bu}_4\text{NPF}_6$ using a 1 mm diameter Pt electrode recorded at 0.1 V s^{-1} to 20.0 V s^{-1} in the potential range of -1.74 V to 1.47 V vs. Fc/Fc^+ . Solid line (—) represents experimental CV data and dotted line (.....) represents digital simulated CV data based on the proposed mechanism and associated electrochemical potentials in Scheme 3.1 and kinetic parameters in Table 3.1. The currents were normalised by multiplying by $\nu^{-1/2}$ to facilitate comparison between all scan rates.

3.2.2. Controlled Potential Electrolysis

The voltammograms shown in Figure 3.2 are similar in appearance to what is expected for a one-electron chemically reversible process (for short duration), which would imply that the oxidation is producing a cation radical ($\text{CAF}^{\bullet+}$) which reacts in the presence of water to form another product at time scales longer than a few seconds. In order to accurately determine the number of electrons transferred during the oxidation of CAF and examine the long term product formed after the oxidation, CPE was performed on CAF in initially dried MeCN. An oxidative potential at 1.95 V vs. Ag wire, ca. 100 mV more positive than the anodic peak, was applied as shown in Figure 3.3, and maintained over a period of time. The CVs of 2 mM CAF in initially dried MeCN containing 0.2 M *n*-Bu₄NPF₆ before and after CPE are shown in Figure 3.3. Over the timescale of the electrolysis experiment the water content of the solvent would be expected to increase, so the final water content could be closer to undried conditions.^[3]

After one electron per molecule was transferred during CPE, the initial anodic peak at 1.85 V vs. Ag wire corresponding to the oxidation of CAF disappeared completely as shown in Figure 3.3 (Green line). This confirms that CAF can be fully oxidised in a one-electron process. In addition, two new sets of reduction peaks were observed at 0.1 V and -0.4 V vs. Ag wire. Further oxidation of the solution resulted in a significant increase in the peak current for the set of peaks at -0.4 V vs. Ag wire but no increase in the peak current for the set of peaks at 0.1 V vs. Ag wire, suggesting that the peaks at -0.4 V vs. Ag wire are due to formation of side products of the solvent oxidation. The species responsible for the peak at 0.1 V vs. Ag wire cannot be the initially formed radical cation ($\text{CAF}^{\bullet+}$) as its peak is too far separated from the oxidation of CAF, and is instead associated with the reduction of a reaction product.

Thereafter, a reductive potential at 0 V vs. Ag wire, ca. 100 mV more negative than the cathodic peak, was applied to the solution in the working electrode compartment containing the oxidised product to examine whether the oxidised product can be reduced back to the starting material. The disappearance of the reversible peak at 0.1 V vs. Ag wire was accompanied by the reappearance of the anodic peak at 1.85 V vs. Ag wire as shown in Figure 3.3 (Blue line). The reappearance of

the anodic peak suggests that the oxidised product could be electrochemically reduced to regenerate CAF, indicating that the oxidised product is structurally similar to CAF.

Figure 3.3: First cycle (Solid line, —) and second cycle (Dotted line,) CV of 2 mM CAF in initially dried MeCN containing 0.2 M $n\text{-Bu}_4\text{NPF}_6$ on a 1 mm diameter Pt planar electrode recorded at 0.1 V s^{-1} before CPE (Red line), after oxidative CPE applied at 100 mV past the anodic peak (Green line), after reductive CPE applied at 100 mV past the cathodic peak (Blue line). All CVs were initially scanned in the positive potential direction indicated by the black arrow from 0 V vs. Ag wire.

Using large-scale electrolysis (20 mM CAF in MeCN) to improve the ability to isolate the oxidised product requires a large amount of supporting electrolyte to reduce ion migration. As a result, isolating the oxidised product via large-scale electrolysis first requires the removal of the electrolyte after electrolysis. While the electrolyte could be separated from the oxidised product by flash column chromatography, it was also found that the oxidised product spontaneously converted back to CAF when it passed through the flash column.

3.2.3. Chemical Oxidation

An alternative way to examine the oxidation process is via chemical oxidation. The advantages of chemical oxidation are that it is fast and does not require electrolyte. The chemical oxidant used in this study was NOSbF_6 because it is a one-electron oxidant and produces gaseous nitric oxide (NO) as the by product of the oxidation process which can be easily removed by purging an inert gas through the solution.^[5] As the bulk electrolysis experiments showed that the oxidation of CAF is a one-electron transfer process, a molar equivalent of NOSbF_6 and CAF were reacted in MeCN. The results from NMR spectroscopic studies (see Appendix 8.1) indicated that the reaction was quantitative with no observable side products. CV experiments performed on the oxidised product showed that it was the same species as detected at the end of the electrolysis reaction (Figure 3.3, Green line), confirming that the chemical and electrochemical oxidation reactions were producing the same product.

The structure of the oxidised product obtained by chemical oxidation was confirmed by single crystal X-ray crystallography with the data included in the Appendix 8.1. The crystal structure indicated that the oxidised product is a protonated CAF cation (CAFH^+), where the nitrogen atom of CAF at N9 was protonated, supported by SbF_6^- as the counter anion and accompanied by two water molecules. Although the X-ray crystal structure of caffeine hydrochloride dihydrate containing a similar cation was previously reported in 1978,^[6] it was obtained by evaporation of a saturated solution containing CAF in hydrochloric acid, a rich proton source. No proton source, however, was added during the chemical oxidation experiment. Hence, the formation of CAFH^+ via chemical oxidation in MeCN was unexpected. Because the variable scan rate CV experiments indicated that the one-electron oxidation of CAF produced $\text{CAF}^{\bullet+}$, the formation of the protonated cation must come about via a hydrogen atom transfer reaction (rather than a proton transfer reaction). The origin of the $\text{H}^\bullet$ atom at this time remains uncertain. Although the electrochemical results indicate that the lifetime of $\text{CAF}^{\bullet+}$ substantially decreased as the water content increased, the hydrogen atom is not necessarily transferred from water as this would also result in the

formation of HO[•] radicals. It is possible that the presence of water aids in the transfer of a hydrogen atom from the solvent.

3.2.4. Digital Simulations

To further understand the reaction mechanism of CAF, digital simulations were performed on the background subtracted CV data from 2.0 mM CAF on a 1 mm diameter Pt working electrode over variable scan rates between 0.1 Vs⁻¹ and 20.0 Vs⁻¹, as shown in Figure 3.2. The simulations were performed based on a proposed mechanism for the oxidation of CAF as shown in Scheme 3.1, with the electrochemical and kinetic parameters obtained from the simulations presented in Scheme 3.1 and Table 3.1, respectively. The match between the experimental data and simulated curves was performed visually. Due to the number of variables that are used in the simulations, an approximate 20 % variation in the parameters in Table 3.1 could be made while still obtaining reasonable good matches between the experimental and simulated voltammograms.

Initially, a reaction mechanism simply involving an electrochemical (E) step followed by a chemical (C) step between CAF^{•+} and a hydrogen atom donor was proposed. However, the reverse peak corresponding to the reduction of CAF^{•+} back to CAF in the simulation could not match the experimental data over all scan rates. In order to obtain improved fits, an additional step involving the equilibration between CAF^{•+} and an intermediate was required. Since no side products were observed in the NMR spectrum from the chemical oxidation experiment, a transient intermediate [CAF(H₂O)^{•+}], CAF^{•+} hydrogen-bonded to a water molecule, was proposed. Based on the CPE data, in which CAFH⁺ could be electrochemically reduced, a mechanism could also be proposed for the reduction of CAFH⁺. An electron would be gained by CAFH⁺ during reduction to yield CAFH[•] and a hydrogen atom could be lost subsequently to form back CAF and ¹/₂ H₂.

Scheme 3.1: Reaction mechanism for the oxidation of CAF (A) and reduction of oxidised product (B) in MeCN with associated electrochemical potentials and kinetic parameters. ^[a] Formal potential, $E^{\circ}_{(1)} = 1.195$ V vs. Fc/Fc^+ .

Table 3.1: Equilibrium and rate constants obtained by digital simulation of CV data for the reaction mechanism given in Scheme 3.1.^[a]

Kinetic Parameters	K_{eq}	$k_f^{[b]}$	$k_b^{[b]}$
Equation (1)	0.70	2.5×10^2	3.6×10^2
Equation (2)	1.0×10^2	1.0×10^2	1.0

^[a] CV data recorded in MeCN with 0.2 M *n*-Bu₄NPF₆ as the supporting electrolyte in the presence of 0.024 M H₂O on a 1 mm diameter Pt electrode at 24 °C, at scan rates between 0.1 V s⁻¹ and 20.0 V s⁻¹. Diffusion coefficient values were 1.7×10^{-5} cm² s⁻¹ for CAF and 1.0×10^{-5} cm² s⁻¹ for H₂O and the hydrogen atom donor.

^[b] Homogeneous rate constants for the forward (k_f) and back (k_b) reactions have units of s⁻¹ and Lmol⁻¹ s⁻¹ for first- and second-order reactions, respectively.

3.3. Conclusion

CAF is oxidised by one-electron in acetonitrile solutions at 1.2 V vs. Fc/Fc^+ to form the cation radical, $\text{CAF}^{\bullet+}$, which reacts with a hydrogen atom donor to form a diamagnetic cation, CAFH^+ . The formation of CAFH^+ occurs via a hydrogen atom transfer rather than a protonation reaction and CAFH^+ can be converted back to CAF by the application of a reducing potential. A cathodic peak corresponding to the reduction of the oxidised product of CAF was only observed in dried MeCN, highlights the importance of recognizing trace water content in solvents used for electrochemical studies. This is especially significant because some compounds can react reversibly or irreversibly with trace water, while others undergo strong hydrogen-bond interactions with trace water, resulting in uniform shifts in the voltammetric waves.^[7]

3.4. References

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Solid-Phase Extraction - Voltammetric Coupled Detection of Caffeine in Acetonitrile

From Chapter 3, caffeine (CAF) was found to undergo a chemically reversible one-electron oxidation on Pt and GC electrodes in MeCN.^[1] This opens up the possibility of using an organic solvent, such as MeCN which has a wide potential window, for the determination of CAF in beverages. As CAF is soluble in aqueous solutions and organic solvents, solid-phase extraction (SPE) of CAF from the aqueous sample solution into an organic solvent could circumvent the problem involving the background response of the aqueous solution while simultaneously removing water soluble interferences present in the sample solution, yet achieving excellent selectivity at the same time. Therefore, in this chapter, a voltammetric method using MeCN as the solvent media for the quantitative determination of CAF in beverages was investigated and optimized. A comparison was made between voltammetric detection of CAF in MeCN and aqueous buffered solution on bare GC electrode.

4.1. Experimental Procedures

4.1.1. Chemicals

Disodium hydrogen phosphate dihydrate and sodium dihydrogen phosphate monohydrate used for making buffered solution were obtained from Merck. Freeze-dried coffee granules (Moccona), freeze-dried decaffeinated coffee granules (Moccona), tea bags (Lipton) and Coffeemate creamer (Nestle) were bought commercially. The caffeinated beverages (Coca-Cola, RedBull and Pokka Japanese Green Tea) and non-caffeinated beverage (Sprite) were bought and used directly from the bottle or can. 0.2 μ m Supor Membrane syringe filters used for filtering sample solutions for voltammetric and HPLC measurements were obtained from Pall.

4.1.2. Preparation of Stock Solution and Beverages

50 mM CAF stock solution in MeCN containing 0.2 M electrolyte was prepared by dissolving CAF (48.54 mg) with 5 mL of a stock solution of 0.2 M electrolyte in MeCN. Various concentrations of CAF in MeCN containing the electrolyte were obtained by mixing appropriate volumes of the CAF stock solution and electrolyte stock solution to make up a 1 mL solution as illustrated in Table 4.1. 0.1 M phosphate buffer stock solution pH 7.2 (PBS) was prepared from disodium hydrogen phosphate dihydrate and sodium dihydrogen phosphate monohydrate. 50 mM CAF stock solution in PBS was prepared by dissolving CAF (48.56 mg) with 5 mL of PBS. Various concentrations of CAF in PBS were obtained by diluting the appropriate volume of the CAF stock solution with PBS to make up a 25 mL solution as illustrated in Table 4.2.

Instant coffee solution was prepared by dissolving freeze-dried coffee granules (1 g) in 100 mL of water, which was estimated from the recommended amount of one tablespoon (3–4 g) in a cup of water (330 mL). Instant coffee solution with added creamer was prepared by mixing creamer (1.51 g) with 50 mL of the prepared instant coffee solution. Decaffeinated coffee solution was prepared by dissolving decaffeinated freeze-dried coffee granules (1 g) in 100 mL of water. Tea

was prepared by soaking the tea satchel in 250 mL of water (95 °C) for 3 min. The various prepared solutions were kept in a refrigerator at 4 °C.

Table 4.1: Respective volume of CAF stock solution and electrolyte stock solution to make up 1 mL of the required CAF concentration in MeCN.

CAF conc. in MeCN / mM	Vol. of CAF stock solution / μL	Vol. of electrolyte stock solution/ μL
0.25	5	995
0.5	10	990
1.0	20	980
2.0	40	960
3.0	60	940
4.0	80	920
5.0	100	900

Table 4.2: Volume of CAF stock solution to be diluted to make up 25 mL of required CAF concentration in PBS.

CAF conc. in PBS/ mM	Vol. of CAF stock solution/ μL
0.05	25
0.10	50
0.15	75
0.20	100
0.25	125
0.30	150

4.1.3. Sample Preparation

Oasis HLB solid-phase extraction cartridges (6 cc, 500 mg Sorbent, 60 μm pore size) were used to extract the CAF from the aqueous sample solutions. A 5-step extraction procedure involving conditioning, equilibrating, sample loading, washing and eluting was adopted as depicted in Scheme 4.1. The cartridge was conditioned with methanol (3 mL) and equilibrated with water (5 mL). The sample solution (5 mL) was loaded into the cartridge and subsequently washed with water (5 mL). MeCN (5 mL) was used to elute CAF from the cartridge into an 8-mL vial containing preweighed supporting electrolyte (388 mg). After each step, air was pushed through the cartridge to remove any excess solvent. The concentration of CAF in the sample solution was assumed to be the same as the eluted solution containing extracted CAF, since identical volumes was used for loading and eluting (this was verified by performing caffeine recovery tests on the SPE procedure and is discussed in more details below). The 5 mL extracted sample solution was separated into five 1 mL solutions and the CAF content of the sample solution was separately determined in each of the five solutions using voltammetric means and taking an average of the five measurements.

Only sample dilution was required for the detection of CAF in aqueous samples. The sample solution (2.5 mL) was diluted with PBS and made up to 25 mL, resulting in a ten-fold dilution. The CAF content was determined by performing five different measurements on the diluted sample solution and taking an average of the five measurements.

Scheme 4.1: Solid-phase extraction procedure

4.1.4. Instrumentation

The mobile phase used for HPLC analysis of CAF consisted of methanol-phosphate buffer pH 3 (30:70 %v/v) with a flow rate of 1.0 mL/min unless otherwise stated and an injection volume of 20 μ L and UV-vis detector set at 268 nm.

4.1.5. Voltammetric Experiments

Square-wave voltammetry (SWV) experiments in MeCN for detection were performed in a 1.5 mL microcentrifuge tube. Conditions used in aqueous solutions were adopted from Khoo et. al. and adjusted accordingly.^[2] A 3 mm diameter planar GC disk working electrode (Metrohm), a Pt wire auxiliary electrode (Metrohm) and an Ag/AgCl reference electrode (Metrohm) were used for voltammetric experiments in aqueous solutions. All sample solutions were spiked with CAF stock solutions to confirm the oxidation potential of CAF. SWV experiments in MeCN were carried out under a step potential of 5 mV and modulation amplitude of 20 mV which had been optimized to obtain the best peak shape and peak height. Differential pulse voltammetry (DPV) experiments in aqueous solutions were carried out under a step potential of 4 mV and modulation amplitude of 25 mV.

4.2. Results and Discussion

4.2.1. SWV of CAF in MeCN and Calibration Plot

The total volume of the MeCN sample solutions was kept at 1 mL and a 1.5 mL microcentrifuge tube was used in order to reduce the usage of the electrolyte and solvent. As all electrodes that were used for the non-aqueous experiments were small (2 to 3 mm in total diameter), they could easily fit into the microcentrifuge tube. (See Appendix 8.2) For purely aqueous samples, the volume was maintained between 10 mL to 25 mL and the experiment conducted in a 50 mL Metrohm glass cell, since there is no significant advantage in using very small volumes.

SWV is a rapid electrochemical method for analysis applicable for species that undergo fast heterogeneous electron transfer, so it was adopted in the quantitative determination of CAF in MeCN to obtain the anodic peak potential and peak height. Voltammograms of CAF in the concentration range of 0.25 to 5.0 mM on GC electrode in MeCN are shown in Figure 4.1. The oxidation peak potential for CAF on GC electrode occurs at ca. 1.2 V vs. Fc/Fc^+ in MeCN. An excellent linear fit was observed for the anodic peak height in the concentration range between 0.25 and 5.0 mM with a high correlation coefficient, R^2 of 0.998 as shown in the inset in Figure 4.1. The small extra peaks at ca. 1.4–1.6 V vs. Fc/Fc^+ that occur after the main oxidation peak are likely due to additional products formed via the oxidation of CAF, since the initially formed caffeine radical cation is very reactive.^[1] A low relative standard deviation of approximately 4 % was obtained for the seven replicates performed between 0.5 and 5.0 mM, while a relative standard deviation of approximately 9 % was obtained for 0.25 mM, suggesting excellent repeatability between 0.5 and 5.0 mM. It should also be noted that the CAF content in most caffeinated beverages lies in the linear concentration range of 0.25 to 5.0 mM. Thus, no sample dilution or preconcentration is required. However, if sample enrichment is needed, it can simply be achieved by increasing the volume of sample solution passed through the SPE cartridge. The percentage recoveries for the SPE procedure were good and found to be between 107.3 % and

117.3 %. The percentage recoveries were calculated using the recovery of eluted CAF from a known concentration of CAF loaded into the SPE cartridge.

Figure 4.1: (Left) SWV of increasing concentration of CAF on GC electrode in MeCN (0 mM, 0.25 mM, 0.5 mM, 1 mM, 2 mM, 3 mM, 4 mM, 5 mM). (Right) Calibration plot of CAF peak height with increasing concentration. Standard deviations marked in red for each concentration were obtained from 7 replicates.

4.2.2. DPV and SWV of CAF in PBS and Calibration Plots

In order to compare the detection capability of CAF in aqueous solutions compared to CAF extracted into MeCN, voltammetric experiments were also performed directly in aqueous solutions. DPV and SWV have previously been used by different groups to detect CAF on 3 mm diameter planar carbonaceous electrodes. Thus, DPV and SWV were performed on a 3 mm diameter planar GC electrode to examine the performance of both methods. A bare GC electrode is seldom used to quantify CAF so the conditions used in this study were adopted from Khoo et al. and adjusted accordingly.^[2] Sample dilution is a common sample preparation for detection of CAF and is performed in order to introduce supporting electrolyte into the sample solution and to maintain the pH within a desired pH range. In this study, sample solutions were diluted with PBS.

Thus, the calibration plot was performed in the concentration range of 0.05 and 0.30 mM, an expected concentration range of caffeinated beverages after ten-fold dilution. Voltammograms of CAF in the concentration range of 0.05 to 0.30 mM on GC electrode in PBS are shown in Figures 4.2(a) and (b) using DPV and SWV, respectively.

The oxidation peak potential of CAF on GC electrode occurs at ca. 1.37 V vs. Ag/AgCl and 1.41 V vs. Ag/AgCl in PBS for DPV and SWV, respectively. A good linear fit was observed for both DPV and SWV in the concentration range of 0.05 to 0.30 mM with high correlation coefficients R^2 of 0.996 and 0.998, respectively as shown in the insets of Figures 4.2(a) and (b). A low relative standard deviation of approximately 3 % was obtained for the seven replicates performed between 0.05 and 0.30 mM for DPV and SWV, suggesting good repeatability could be obtained for concentrations between 0.05 and 0.30 mM.

Figure 4.2: (a) DPV and (b) SWV of increasing concentration of CAF on GC electrode in PBS (0 μM , 50 μM , 100 μM , 150 μM , 200 μM , 250 μM , 300 μM). (Insets): Calibration plot of CAF peak height with increasing concentration. Standard deviations marked in red were obtained from 7 replicates.

4.2.3. SWV of Pre-filtered Extracted Real Beverage Samples in MeCN

In order to quantify CAF in real samples, CAF present in the aqueous sample solutions was extracted using SPE followed by elution using MeCN. The solvent medium was thus changed from an aqueous solution into an organic medium while retaining CAF from the sample solution and removing unwanted water-soluble interferences and insoluble non-polar compounds. Aggregates that collected at the bottom of the vial during the elution step could be due to non-polar compounds retained in the extraction cartridge and subsequently eluted out with MeCN. CAF was not detected by HPLC in the eluate after the sample solution had passed through the SPE cartridge during sample loading and washing, indicating that all of the CAF was retained in the SPE cartridge (see Appendix 8.2).

Solid particles present in samples had to be filtered off before they were introduced into the HPLC analytical column to determine the CAF content. In order to ensure there were no discrepancies in the CAF content of sample solutions obtained from the voltammetric and HPLC methods, voltammetric measurements were performed on sample solutions that were pre-filtered and unfiltered prior to extraction.

SWV of the pre-filtered extracted samples on GC electrode in MeCN containing the supporting electrolyte are shown in Figure 4.3. It was observed that the anodic peak potential of CAF in extracted caffeinated sample lies between 1.2 V and 1.26 V vs. Fc/Fc^+ . Furthermore, no peak at 1.2 V vs. Fc/Fc^+ was observed for Sprite, a non-caffeinated soft drink (Figure 4.3(b), Solid line). Spiking of the extracted sample with CAF stock solution showed an increase in the peak height corresponding to CAF (Figure 4.3(b), Dotted line). This suggests that the peak potential between 1.2 V and 1.26 V vs. Fc/Fc^+ is indicative of CAF.

Figure 4.3: (a) Coca-Cola, (b) Sprite, (c) Pokka Green Tea, (d) RedBull, (e) Instant coffee, (f) Instant coffee with creamer, (g) Decaffeinated coffee and (h) Lipton Tea. SWV of pre-filtered extracted sample solution (Solid line, —) and after spiking with CAF stock solution (Dotted line,) on GC electrode in MeCN. All voltammograms were adjusted to the same scale to facilitate comparison between all sample solution.

The CAF content of sample solutions that were pre-filtered and unfiltered prior to extraction were determined from the calibration plot above and compared with results obtained from HPLC measurements and are tabulated in Table 4.3. The results indicated that filtration does not affect the CAF content significantly. This implies that extraction could be performed on unfiltered solutions.

The CAF content of the extracted sample solutions obtained via voltammetric means was in reasonable agreement within 30 % of the values obtained by HPLC. The CAF content in decaffeinated coffee solutions was reported to be between 0 % and 0.3 % which agrees with the HPLC measurements. However, the CAF content obtained by voltammetric means was overestimated by about 130 % compared to the HPLC measurement. This is likely because the CAF content is close to the limit of detection of 0.251 mM (S/N=3).

4.2.4. DPV and SWV of Real Beverage Samples in PBS

DPV and SWV of the diluted aqueous sample solutions on GC electrode in PBS are shown in Figures 4.4 and 4.5, respectively. It was observed that the anodic peak potential of CAF using DPV and SWV for aqueous caffeinated samples in PBS lies between 1.36 and 1.49 V vs. Ag/AgCl and between 1.41 and 1.44 V vs. Ag/AgCl, respectively. No peaks at 1.37 V vs. Ag/AgCl using DPV and 1.41 V vs. Ag/AgCl using SWV were observed for Sprite and decaffeinated coffee solution.

The CAF content of the aqueous samples in PBS determined using DPV and SWV was underestimated by approximately 20 % to 70 % and approximately 50 % to 90 % respectively, compared to HPLC measurements, even though most measurements were found to be well above the detection limit of 0.0196 mM (S/N=3) for DPV and 0.0178 mM (S/N=3) for SWV. Although DPV is a slower method compared to SWV, DPV appears to provide a better result compared to SWV for the detection of CAF in aqueous solutions.

Despite the simple sample preparation for aqueous sample solutions involving only sample dilution, there are some disadvantages. Dilution of the sample solutions reduces the CAF content of low CAF content samples such as decaffeinated coffee, such that CAF can no longer be reliably detected by voltammetric means, as seen in Figures 4.4(g) and 4.5(g). Furthermore, there might be compounds present in the sample solutions that result in a high background current such as for the RedBull solution shown in Figures 4.4(d) and 4.5(d). Hence, the CAF anodic peak was obscured, resulting in CAF not being detected for the RedBull solution despite its rather high reported CAF content.

Table 4.3: Determination of CAF in beverages using voltammetric methods in MeCN ^a and PBS ^a compared to HPLC.

Sample	SWV in MeCN (filtered)	SWV in MeCN (unfiltered)	DPV in PBS	SWV in PBS	HPLC
Coca-Cola ^b	0.129 (± 1)	0.137 (± 2)	0.070 (± 3)	0.008 (± 2)	0.099
Sprite ^b	N. D. ^c	N. D. ^c	N. D. ^c	N. D. ^c	N. D. ^c
Pokka Green Tea ^b	0.129 (± 2)	0.123 (± 5)	0.079 (± 4)	0.020 (± 6)	0.100 ^f
RedBull ^b	0.346 (± 1)	0.357 (± 2)	0.119 (± 9)	ND ^c	0.313
Instant Coffee ^c	40.5 (± 2)	41.3 (± 1)	20.4 (± 2)	16.1 (± 3)	44.7
Instant Coffee with Creamer ^c	37.5 (± 7)	37.6 (± 3)	13.5 (± 3)	11.0 (± 5)	42.8
Decaffeinated Coffee ^c	5.7 (± 5)	5.9 (± 17)	N. D. ^c	N. D. ^c	2.5 ^f
Lipton Tea ^d	54.3 (± 4)	56.4 (± 5)	37.3 (± 5)	25.4 (± 3)	54.1

[a] Five replicate measurements were made on each sample solution. Values in paranthesis are % RSD.

[b] Concentration measured in mg/mL.

[c] Concentration measured in mg/g.

[d] Concentration measured in mg/satchel.

[e] N. D.: Not detected.

[f] Mobile phase: methanol-phosphate buffer pH 3 (20:80 %v/v)

Figure 4.4: (a) Coca-Cola, (b) Sprite, (c) Pokka Green Tea, (d) RedBull, (e) Instant coffee, (f) Instant coffee with creamer, (g) Decaffeinated coffee and (h) Lipton Tea. DPV of ($10 \times$) diluted aqueous samples (Solid line, —) and after spiking with CAF stock solution (Dotted line,) on GC electrode in PBS. All voltammograms were adjusted to the same scale to facilitate comparison between all sample solution.

Figure 4.5: (a) Coca-Cola, (b) Sprite, (c) Pokka Green Tea, (d) RedBull, (e) Instant coffee, (f) Instant coffee with creamer, (g) Decaffeinated coffee and (h) Lipton Tea. SWV of (10 ×) diluted aqueous samples (Solid line, —) and after spiking with CAF stock solution (Dotted line,) on GC electrode in PBS. All voltammograms were adjusted to the same scale to facilitate comparison between all sample solution.

4.2.5. SPE Coupled to Voltammetry Compared with Direct Voltammetric

Determination of CAF

The advantages of using the SPE-voltammetric (SPE-V) method for the determination of CAF transferred into MeCN can be discussed in comparison to using a non-SPE direct voltammetric (direct-V) method for the detection of CAF in aqueous samples without any extraction procedure.

Various electrochemical studies have highlighted the very low detection limits of using modified electrodes for the direct-V determination of CAF in aqueous solutions.^[2,3] However, the SPE-V method described in this report can also be used to lower the detection limits by concentrating the sample (injecting more solution through the SPE cartridge). Nevertheless, for the common caffeinated drinks examined in this study, the levels were of a range that required no preconcentration.

Most recent direct-V studies use chemically modified electrodes to obtain the desired sensitivity which requires an electrode pre-treatment step (which may not be completely reproducible).^[3] Furthermore, depending on whether adsorption occurs, the electrodes may need to be freshly treated for each scan. The SPE-V procedure takes approximately 30 minutes to prepare the sample for analysis, but no special electrode treatment procedure is required. HPLC methods also require sample pretreatment in order to remove particulates that can damage the column and the analysis time is longer than either the SPE-V or direct-V methods.

The biggest disadvantage of the direct-V method is the presence of any compounds that undergo oxidation at a similar potential to CAF (which appeared to occur in the case of RedBull samples analysed by direct-V as shown in Figures 4.4(d) and 4.5(d)). The presence of species in the sample that can foul the electrode is also problematic, such as can occur for samples containing milk proteins (which are removed using the SPE cartridges). Therefore, both the SPE-V and HPLC methods have the advantage of being able to remove or separate potentially interfering components of the matrix.

The absolute costs are difficult to assess between different studies but it would be expected that both the electrochemical methods (SPE-V and direct-V) are more economical than HPLC methods, both in terms of equipment and reagents. The SPE-V method requires the use of SPE cartridges and a relatively small quantity of MeCN, but is otherwise similar to the direct-V method (which requires the electrode coating agents).

Using MeCN rather than pure water is less environmentally friendly, but the amount can be kept relatively low (a few mL per sample). Both electrochemical methods use less organic solvents than the HPLC procedure (which requires methanol).

4.3. Conclusion

Using solid-phase extraction, CAF in beverages could be extracted into the organic medium, MeCN. In the process, water-soluble interferences and insoluble non-polar compounds could be removed. The detection of CAF on GC electrodes in MeCN was performed in a 1.5 mL microcentrifuge tube, reducing the amount of electrolyte and solvent used. The CAF content obtained using this method provided similar accuracy when compared to the results obtained from HPLC. Furthermore, the extraction process is simple and the detection process is convenient and economical compared to HPLC. The direct detection of CAF in aqueous solutions was also performed for comparison purposes. The SPE method has the advantage that it is able to remove other contaminants that might voltammetrically interfere with the determination of CAF, as well as allowing preconcentration to be performed for samples that contain very low levels of CAF.

4.4. References

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Electrochemical Behaviour of Capsaicin in Acetonitrile

Previous electrochemical studies on capsaicin (CAPH) reported that capsaicin adsorbed onto the electrode after oxidation in aqueous solution.^[1] The intermediate formed was proposed to undergo hydrolysis with water to generate a *o*-quinone moiety, which can give a reversible redox response.^[1b] In this chapter, the electrochemical behaviour of CAPH was examined using various electrochemical methods in the aprotic organic solvent acetonitrile under low and high water content. Low water content can reduce the tendency for any unwanted chemical reaction and enable the study of the intermediate formed, while products formed due to chemical reaction with water under high water content can be identified. The abbreviation of capsaicin (CAPH) will be used later in the chapter to facilitate in the mechanistic explanation.

5.1. Experimental Procedures

5.1.1. Chemicals

Ammonium formate solution (10 M in water) used in CPE experiments for identification of electrolysed products was purchased from Fluka.

5.1.2. Instrumentation

Waters[®] ACQ-TQD triple quadrupole mass spectrometer (Milford, MA, USA) was used in this chapter for the identification of the electrolysed products. Electrospray ionization (ESI) in negative polarity mode (-) was utilized as the ionization source. Samples were directly injected into the mass spectrometer with a scan range of 4-2000 Da. The ACQ-TQD system was calibrated and tuned prior to the analysis by using sodium cesium iodide (NaCsI) tuning solution (Sigma Aldrich). Argon was used as the desolvation gas in the ionization source. Optimized ionization source and mass analyzer parameters are given in Table 5.1 and Table 5.2, respectively.

5.1.3. Voltammetric Experiments

A wet solution with high water content used in CPE was prepared by adding a certain amount of water of desired concentration to the volumetric flask containing the analyte and the supporting electrolyte before making up to the required volume with MeCN.

Rotating disk electrode voltammetric experiments were performed using a Metrohm Autolab RDE-2 rotator together with a 3 mm diameter planar glassy carbon electrode (Metrohm) in a three-electrode electrochemical cell.

Table 5.1: Ionization source settings

ESI Source	Settings
Capillary voltage (kV)	3.00
Cone voltage (V)	62.00
Extractor voltage (V)	3.00
Radio Frequency (RF) voltage (V)	0.10
Source Temperature (°C)	120
Desolvation Temperature (°C)	250
Desolvation Gas Flow (L/h)	500
Collision Gas Flow (mL/min)	0.10

Table 5.2: MS analyzer mode settings for MS scan mode

Analyzer	Settings
Low Mass (LM) 1 resolution	15.00
High Mass (HM) 1 resolution	15.00
Ion energy (eV)	1.00
MS mode entrance energy (eV)	50.00
MS mode collision energy (eV)	3.00
MS mode exit energy (eV)	50.00
MSMS mode entrance energy (eV)	1.00
Low Mass (LM) 2 resolution	15.00
High Mass (HM) 2 resolution	15.00
Ion energy 2 (eV)	1.00
Gain	1.00
Multiplier	-589.62

5.2. Results and Discussion

5.2.1. Voltammetry of CAPH in MeCN

Phenolic compounds tend to undergo hydrogen bonding with water in the solvent, which might cause the oxidation peaks to shift to less positive potentials.^[2] In order to examine the effect water has on the oxidation of CAPH, CV was performed on CAPH in dried MeCN and with successive additions of water. As glassy carbon (GC) has a higher overpotential for hydronium ion reduction compared to Pt, a GC electrode was used throughout all experiments. The CVs of CAPH in MeCN with increasing additions of water are shown in Figure 5.1. CAPH displayed two chemically irreversible anodic peaks at ca. 0.7 V and 1.0 V vs. Fc/Fc^+ under dried conditions, with a low initial water content of about 6 mM. Two additional cathodic peaks at ca. 0.1 V and -0.4 V vs. Fc/Fc^+ can be seen on the reverse scan. As the water content increased, the anodic peak at 1.0 V vs. Fc/Fc^+ shifted to less positive potential and eventually merged with the first peak at water content of about 1 M, with a doubled overall peak current. The cathodic peak at 0.1 V vs. Fc/Fc^+ that was detected on the reverse scan was observed to shift to less positive potentials, with an increase in peak height as the water content increased. It is clear that the voltammetry of CAPH is affected by the water content in MeCN. It was also rather surprising to observe two oxidation processes in dried MeCN, given that most phenolic compounds only undergo an oxidation process involving two electrons.^[2] As such, further experiments on CAPH were performed in MeCN in both dried and wet conditions with a low and high water content of about 5-10 mM and 1 M, respectively.

Figure 5.1: CV of 2 mM CAPH in dried MeCN (Red) containing 0.2 M $n\text{-Bu}_4\text{NPF}_6$ using 1 mm diameter GC electrode recorded at scan rate 0.1 V s^{-1} with increasing additions of water to the solution. Water content (lowest to highest): 5.8 mM, 39.1 mM, 116.6 mM, 282.1 mM, 555.8 mM, 1095.0 mM, 2142.3 mM, 3639.8 mM, 5055.8 mM.

The correlation between the two oxidation processes and the two cathodic peaks was examined using CV by changing the switching potential of the forward scan as shown in Figure 5.2. When the switching potential was limited to the first oxidation process, only the cathodic peak at ca. $-0.5 \text{ V vs. Fc/Fc}^+$ was observed, whereas when the switching potential was applied past the second oxidation process, both cathodic peaks at 0.1 V and $-0.5 \text{ V vs. Fc/Fc}^+$ were present. This indicates that the cathodic peak at $0.1 \text{ V vs. Fc/Fc}^+$ is related to a product formed only after the second oxidation process, while the cathodic peak at $-0.5 \text{ V vs. Fc/Fc}^+$ is attributed to a product formed after the first oxidation process.

Figure 5.2: CV of 2 mM CAPH in dried MeCN containing 0.2 M $n\text{-Bu}_4\text{NPF}_6$ using 1 mm diameter GC electrode recorded at scan rate 0.1 V s^{-1} . Switching potential limited to first oxidation process (Dotted line,), switching potential applied past second oxidation process (Solid line, —).

As capsaicinoids are structurally similar with variations only at the aliphatic chain, the effects of the variations on the CV profile was examined by studying both CAPH and nonivamide. Nonivamide differs from CAPH by the alkene functional group and the branched chain. Yet, the CVs of both CAPH and nonivamide as shown in Figure 5.3 were very similar. Hence, it can be expected that they undergo similar oxidation process. Thus, their electrochemical behaviour is independent of the differences at the aliphatic chain.

Figure 5.3: CV of 2 mM CAPH (Left) and 2 mM nonivamide (Right) in dried MeCN containing 0.2 M $n\text{-Bu}_4\text{NPF}_6$ using 1 mm diameter GC electrode recorded at a scan rate of 0.1 V s^{-1} . Both voltammograms were adjusted to the same scale to facilitate the comparison of the anodic peak current.

5.2.2. Voltammetry of CAPH with Varying Scan Rates

The oxidation of CAPH was performed in various scan rates ranging from 0.1 V s^{-1} to 20.0 V s^{-1} in both dried and wet MeCN to observe the changes in the electrochemical behaviour at different timescales and the CV results are depicted in Figure 5.4. The voltammetric profiles did not change significantly over the range of scan rates examined for dried MeCN. In wet MeCN, the cathodic peak at $-0.1 \text{ V vs. Fc/Fc}^+$ diminished as the scan rate increased and disappeared at scan rates $\geq 5.0 \text{ V s}^{-1}$. This indicates that the cathodic peak at $-0.1 \text{ V vs. Fc/Fc}^+$ is probably due to the reduction of a product formed by the initially oxidised species reacting with water.

In both dried and wet MeCN, the anodic peak current, i_p , is directly proportional to square root of scan rate with a high correlation coefficients of 0.994 and 0.999 respectively, as shown in Figure 5.4, suggesting that the oxidation process was diffusion-controlled regardless of the water content. However, the Randles-Sevcik equation used for describing diffusion-controlled processes

primarily applies to straightforward electron transfer reactions, and may not be applicable for coupled chemical processes.

Figure 5.4: Normalised CV of 2 mM CAPH in (a) dried and (b) wet MeCN containing 0.2 M $n\text{-Bu}_4\text{NPF}_6$ using 1 mm diameter GC electrode recorded with increasing scan rate ranging from 0.1 V s^{-1} to 20.0 V s^{-1} with its corresponding plot of current against square root of scan rate (Right). The currents were normalised by multiplying by $v^{-1/2}$ to facilitate comparison between all scan rates.

5.2.3. Controlled Potential Electrolysis

In order to determine the number of electrons transferred during the oxidation of CAPH and to examine the long term product(s) formed after oxidation, CPE was performed in MeCN under both undried and wet conditions. The CV of 2 mM CAPH in initially dried MeCN containing 0.2 M *n*-Bu₄NPF₆ (Figure 5.5, Red line) showed that two anodic peaks at 0.7 V and 1.0 V vs. Fc/Fc⁺ were observed before CPE was performed. Over the timescale of the electrolysis experiment the water content of the solvent would be expected to increase, so the final water content could be closer to undried conditions.^[3] An oxidative potential at 0.8 V vs. Fc/Fc⁺, ca. 100 mV more positive than the first anodic peak, was applied and maintained over a period of time. A total of 1.87 electrons were transferred for the first oxidation process (see Appendix 8.3), suggesting that the first oxidation process could involve two electrons. After the oxidation, the anodic peak at 0.7 V vs. Fc/Fc⁺ disappeared and the growth of a cathodic peak at 0.1 V vs. Fc/Fc⁺ was observed (Figure 5.5, Green line). Another oxidative potential at 1.1 V vs. Fc/Fc⁺, ca. 100 mV more positive than the second anodic peak was applied and maintained over a period of time. A total of 2.16 electrons were transferred for the second oxidation process (see Appendix 8.3), also indicating that the second oxidation process involved the transfer of two electrons over electrolysis timescales. After the oxidation, the anodic peak at the 1.0 V vs. Fc/Fc⁺ disappeared and a cathodic peak at -0.1 V vs. Fc/Fc⁺ became more prominent (Figure 5.5, Blue line). Thus, the electrolysis experiments confirmed that the cathodic peak at -0.1 V vs. Fc/Fc⁺ was due the reduction of the oxidised product.

The CV of 2 mM CAPH in wet MeCN containing 0.2 M *n*-Bu₄NPF₆ (Figure 5.6, Red line) showed an anodic peak at 0.6 V vs. Fc/Fc⁺ and a cathodic peak was observed at -0.1 V vs. Fc/Fc⁺ on the reverse scan before CPE was performed (Figure 5.6, Red line). An oxidative potential at 0.8 V vs. Fc/Fc⁺, ca. 200 mV more positive than the anodic peak was applied and maintained over a period of time. A total of 2.02 electrons were transferred for the exhaustive electrolysis (see Appendix 8.3), indicating that the oxidation process could involve two electrons. After the

oxidation, the anodic peak at 0.6 V vs. Fc/Fc^+ disappeared. A cathodic peak at -0.1 V vs. Fc/Fc^+ was observed corresponding to the reduction of the oxidised product (Figure 5.6, Blue line).

Figure 5.5: CV of 2 mM CAPH in initially dried MeCN (10-25 mM H₂O) containing 0.2 M *n*-Bu₄NPF₆ using 1 mm diameter GC electrode recorded at a scan rate of 0.1 V s⁻¹. Before CPE (Red line), after oxidative CPE applied at 100 mV past the first anodic peak (Green line), after oxidative CPE applied at 100 mV past the second anodic peak (Blue line). Solid (—) and dotted (.....) line denotes the direction of the potential scan in the positive potential direction and in the negative potential direction respectively from 0.4 V vs. Fc/Fc⁺.

Figure 5.6: CV of 2 mM CAPH in wet MeCN (1 M H₂O) containing 0.2 M *n*-Bu₄NPF₆ using 1 mm diameter GC electrode recorded at a scan rate of 0.1 V s⁻¹. Before CPE (Red line), after oxidative CPE applied at 200 mV past the anodic peak (Blue line). Solid (—) and dotted (.....) line denotes the direction of the potential scan in the positive direction and in the negative direction respectively from 0.2 V vs. Fc/Fc⁺.

CPE under undried and wet conditions led to a different total number of electrons transferred. In undried conditions, two oxidation processes were observed, which corresponded to the transfer of a total of four electrons. In wet conditions, only one oxidation process was observed, which involved the transfer of two electrons in total. Despite bulk electrolysis being one of the ways to determine the number of electrons transferred for an electrochemical process, the timescale ranging from minutes to hours can allow the oxidation of side products to occur. Furthermore, it is difficult to maintain the dried condition throughout the duration of the CPE. Since the water content in wet MeCN is rather high, the increase in water content during the CPE experiment would be lower. Knowing that a transfer of two electrons was observed in wet MeCN coupled with the knowledge from earlier in section 5.2.1. that the oxidation peak current doubled after the two oxidation processes merged with higher water content, it is probable that the two oxidation processes involved a transfer of one electron each in dried MeCN.

Peak current obtained from the CV of several compounds with well-known electrochemistry (catechol, vanillin and tocopherol acetate) was also compared with that of capsaicin as an alternative to determine the number of electrons transferred, as the timescale is in the range of seconds. Catechol and vanillin were chosen due to the similarity to the vanillyl moiety in CAPH, while tocopherol acetate was chosen due to the presence of the aliphatic chain similar to CAPH. The oxidation of catechol and vanillin are known to involve two electrons,^[4] while tocopherol acetate is known to undergo a one-electron oxidation.^[2d] Despite slight expected differences in the diffusion coefficients, the peak current of the abovementioned compounds in Figure 5.7 is almost doubled that of the first anodic peak current of CAPH, indicating that the first oxidation process of CAPH in dried MeCN involved only one electron.

Figure 5.7: CV of 2 mM catechol (Left), 2 mM vanillin (Centre) and 2 mM tocopherol acetate (Right) with 2 mM CAPH (Dotted line, “----”) in MeCN containing 0.2 M *n*-Bu₄NPF₆ using 1 mm diameter GC electrode recorded at a scan rate of 0.1 V s⁻¹. All voltammograms were adjusted to the same scale to facilitate the comparison of the anodic peak current.

An attempt to isolate the products of oxidation after bulk electrolysis did not yield good results. Due to the nature of bulk electrolysis, a large amount of supporting electrolyte compared to analyte is required to reduce ion migration during the electrolysis. As a result, direct characterisation of the products formed by NMR was impeded by the large amount of supporting electrolyte present. Identification of products formed from electrochemical means by mass-spectrometry (MS) has been successful in other studies^[5] and was used in this section of the study. The supporting electrolyte used in organic solvents in this study, *n*-Bu₄NPF₆, is bulky and non-volatile. It gives strong background peaks in mass spectra, causes crystallisation on the electrospray needle or curtain plate and can cause the analyte ion suppression in the ionisation chamber and is usually not recommended.^[6] Therefore, ammonium formate was used, which is a supporting electrolyte that has been recommended for use in electrochemical-mass spectrometry system due to its volatility. A mixture of water (4.5 M) and ammonium formate (20 mM) in MeCN was prepared to make the electrolyte solution. The use of water was instrumental in dissolving ammonium formate, but it was kept to the lowest possible level to ensure the solvent was still mostly organic and could replicate the similar environment CAPH is exposed to throughout the study. The amount of ammonium formate was also kept low to allow a better analyte signal. As the grade of CAPH used in this experiment was natural grade, it consists of a

mixture of capsaicinoids (capsaicin, dihydrocapsaicin and nordihydrocapsaicin). Thus, nonivamide, one of the capsaicinoids, of a higher purity (> 96 %) was used to allow a better monitoring of the starting material and products formed after oxidation. The CV of 2 mM nonivamide in a mixture of water (4.5 M) and MeCN containing ammonium formate (20 mM) showed an anodic peak at 0.7 V vs. Ag wire in Figure 5.8 (Solid Line). An oxidative potential at 0.8 V vs. Ag wire, ca. 100 mV more positive than the anodic peak was applied and maintained over a period of time. A total of 1.58 electrons was transferred during the electrolysis with the potential held after the anodic peak (see Appendix 8.3). The CV after the bulk oxidation showed that the anodic peak has partly diminished as shown in Figure 5.8 (Dotted line). The solution containing the oxidised product was withdrawn for MS characterisation. A MS peak with m/z ratio of 583.27 was identified after CPE, suggesting a dimeric product. (see Appendix 8.3) Although the identity of the product could not be deduced from the fragmentation pattern, dimeric products were considered as a possibility after the oxidation of CAPH. Dimeric products were also reported by oxidation of CAPH via enzymatic produced hydroxyl radicals,^[1a, 7] oxidation of CAPH in linoleic acid in the presence of a radical initiator^[8] as well as via photochemical means.^[9]

Figure 5.8: CV of 2 mM nonivamide in a mixture of water (4.5 M) and MeCN containing 20 mM ammonium formate using 1 mm diameter GC electrode recorded at a scan rate of 0.1 V s^{-1} . Before CPE (Solid line, —), after oxidative CPE applied at 100 mV past the first anodic peak (Dotted line,).

5.2.4. Rotating Disk Electrode Voltammetry

In order to better understand the nature of the second oxidation process as well as have a better estimate of the diffusion coefficient of CAPH in MeCN, linear sweep voltammetry on a rotating disk electrode was used on nonivamide. Only dried MeCN was used in this section in order to examine the second oxidation process as a better separation between the two oxidation processes was observed when the water content is low.

Linear sweep voltammograms of nonivamide with increasing rotation speed from 200 rpm to 5000 rpm at a scan rate of 0.02 V s^{-1} were depicted in Figure 5.9. The voltammograms observed have the typical sigmoidal curve observed with a rotating disk electrode. The current increased sharply as the potential applied approaches the half-wave potential. A gentle slope can be seen between 0.6 V and 0.8 V vs. Fc/Fc^+ even at high rotational speed, before the current increased sharply again and levelling off at a limiting current which was dependent on the rotation speed. This observation points towards a second electron transfer step taking place from the molecule and not from a product formed after oxidation. This is because any product formed after the first electron oxidation would immediately be swept away from the rotating electrode, resulting in only one plateau.

With the number of electrons transferred on the short voltammetric timescale for the first oxidation process suggested to be one (based on the earlier comparisons with other molecules), a comparison of the limiting current observed for the first process and the second process indicated that the second electron transfer also likely involved the transfer of one electron. With the knowledge of the number of electron transferred, the limiting current i_L , as well as the rotational speed of the electrode ω , the diffusion coefficient of nonivamide in MeCN could be determined from Levich equation in equation (2.3) in Chapter 2.

The limiting current after the second electron transfer step was used in the calculation for the diffusion coefficient due to the constant current observed after the second oxidation process. A plot of the limiting current with the square-root of the rotation speed (in rad s^{-1}) yielded a best fit

line with a gradient of $1.42 \times 10^{-5} \text{ A s}^{1/2}$ and a high correlation coefficient of 0.999 as illustrated in Figure 5.9. A diffusion coefficient of $6.30 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ calculated from the gradient was obtained for nonivamide in MeCN.

Figure 5.9: Linear sweep voltammogram of 2 mM nonivamide with increasing rotation speed (Left) in dried MeCN containing 0.2 M *n*-Bu₄NPF₆ using 3 mm GC electrode recorded at a scan rate of 0.02 V s^{-1} . A plot of limiting current at various rotational speed of the electrode against the square root of the rotational speed (Right).

5.2.5. Proposed Mechanism

Based on the results that were presented earlier, a mechanism was proposed for the electrochemical oxidation of CAPH in MeCN under dried and wet conditions as illustrated in Scheme 5.1. The observation of a diffusion controlled electrochemical oxidation process in MeCN suggested that the mechanism involved should deviate slightly from that reported in aqueous solutions. CAPH in dried MeCN likely underwent an electrochemical oxidation process via an ECE mechanism similar to phenols, where E represents an electron transfer step and C represents a chemical step. The initial E-step produced a cation radical which then loses a proton to form a phenoxyl radical CAP[•], followed by a further E-step to form a phenoxonium cation CAP⁺. The protons on 2-methoxy could conceivably undergo hydrogen bonding interactions with the phenolic oxygen atom, forming a cyclized ring structure (Scheme 5.1). This stabilised phenoxyl radical formed following deprotonation made the second electron transfer step more difficult to occur (so occurring at a higher potential). This is in contrary to what is usually observed for phenols where the oxidation of the phenoxyl radical occurs at a lower potential than the starting material.^[2]

Increasing the water content of MeCN saw the shift of the second oxidation process at 1.0 V vs. Fc/Fc⁺ to less positive potential, which can be rationalised by the phenoxyl radical undergoing hydrogen bonding with water, which has been shown to lower the oxidation potential of phenols.^[2d] This shift in potential caused by hydrogen bonding was also observed in the electrochemical reduction of quinones as well as the electrochemical oxidation of α -tocopherol resulting in the shift of their reduction and oxidation potentials respectively.^[2c, d]

The cathodic peak detected at 0.1 V vs. Fc/Fc⁺ in low water content (and 0 V vs. Fc/Fc⁺ in high water content) is rather far from the oxidation peak (ca. 600-700 mV). Hence, the reduction cannot be of the phenoxyl radical or cation, but a product formed after the oxidation. As this cathodic peak was only observed after the second oxidation process, the highly reactive phenoxonium cation CAP⁺ formed could have undergone a hydrolysis reaction with water to yield a hemiketal CAPQ(OH), which liberates methanol to yield an *o*-quinone product. Such

observation was also reported in the electrochemical study of vanillin in MeCN and the reduction potential reported was also very similar to that observed in this study.^[4c] At higher scan rates, the hydrolysis reaction was outrun and another cathodic peak between -0.5 V and -0.8 V vs. Fc/Fc^+ under wet conditions was observed. A similar set of peaks were observed between -0.4 V and -0.7 V vs. Fc/Fc^+ under dried conditions. As this cathodic peak was observed when the switching potential was limited to the first oxidation peak, it was believed to be a product formed after the formation of the phenoxyl radical $\text{CAP}^\bullet$. Based on the MS results of the electrolysis of nonivamide in a mixture of water and MeCN containing ammonium formate, a dimeric product formed after oxidation was considered a possibility. Thus, the cathodic peak found around -0.5 V and -0.8 V vs. Fc/Fc^+ was attributed to be the reduction of a dimeric product formed after oxidation.

Scheme 5.1: Proposed electrochemical oxidation mechanism of CAPH in MeCN.

5.3. Conclusion

CAPH undergoes a chemically irreversible oxidation with the observation of two distinct one-electron oxidation peaks in dried MeCN and one two-electron oxidation peak in wet MeCN. A hydrogen bonding interaction between the protons on 2-methoxy and the phenolic oxygen atom possibly stabilised the phenoxyl radical formed after the first oxidation process, due to the formation of a cyclized ring structure, thereby making the second electron transfer step more difficult to proceed. However, in the presence of excess water in solution, the hydrogen bonding interactions with water competed with the hydrogen bonding through the methoxy group, which resulted in the second oxidation wave merging with the first. The highly reactive phenoxonium cation CAP^+ formed after two-electron oxidation underwent a hydrolysis reaction with water to form a hemiketal $CAPQ(OH)$, which quickly convert to form an *o*-quinone intermediate, resulting in the cathodic peak at ca. 0 V vs. Fc/Fc^+ . A dimeric product was identified in the MS analysis of the solution after CPE of nonivamide in a mixture of water and MeCN containing ammonium formate. The cathodic peak at ca. -0.4 V to -0.7 V vs. Fc/Fc^+ in dried conditions and ca. -0.5 V to -0.8 V vs. Fc/Fc^+ in wet conditions could possibly be attributed to the reduction of a dimeric product formed after CPE.

The electrochemical behaviour of CAPH in organic solvent such as MeCN is different from that observed in aqueous solution. The oxidation of CAPH in MeCN is diffusion-controlled, while the oxidation of CAPH in aqueous solution is adsorption-controlled. The adsorption of CAPH onto the electrode in aqueous solution possibly limited the intermediates observed, as compared to in MeCN. Furthermore, the use of organic solvent allowed the electrochemical study of CAPH in varying water content, which showed the influence trace water has on the oxidation potential as well as the electrochemical oxidation mechanism.

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Solid-Phase Extraction - Voltammetric Coupled Detection of Capsaicinoids in Acetonitrile

From Chapter 5, capsaicin was found to undergo two one-electron oxidation process on GC electrode in dried acetonitrile. Incorporating the detection method developed in Chapter 4 for the detection of caffeine,^[1] the detection of capsaicinoids in acetonitrile was examined and optimised in this chapter. The extraction capability of various extraction solvents and simplification of extraction method were also studied. The abbreviation of capsaicinoids (CAPs) will be used later in the chapter to differentiate between capsaicin (CAPH) and the collective term capsaicinoids.

6.1. Experimental Procedures

6.1.1. Chemicals

Paprika Ground powder and Cayenne Pepper Ground powder were obtained commercially from MasterFoods. Tabasco Pepper Sauce was obtained commercially from McIlhenny Company (Avery Island, LA, USA). Fresh red chillies were bought locally from a supermarket.

6.1.2. Preparation of Stock Solution and Samples

A 17,500 ppm CAPs stock solution in MeCN was prepared by dissolving CAPs (68.89 mg) in MeCN (5 mL). Various concentrations of CAPs in MeCN containing 0.2 M electrolyte for the SWV calibration plot were prepared by diluting appropriate volumes of the CAPs stock solution with a 0.2 M stock electrolyte solution to make up a 5 mL solution as illustrated in Table 6.1. The red chilli seed and skin samples were obtained from a total of five chillies. The chilli was sliced open and the seeds were separated from the skin. The seeds were then pounded and ground into a flaky-like solid while the skin was cut into smaller pieces. (See Appendix 8.4.)

6.1.3. Sample Preparation

Two different extraction approaches were used in this study. Extraction procedure A adopted from Kachoosangi et. al.^[2] had three steps, sonication for ten minutes, followed by stirring for two hours and centrifugation for twenty minutes at 10,000 rpm. Extraction procedure B was simplified to only the first step, sonication for ten minutes. Approximately 1 g of each sample was soaked in an extraction solvent (10 mL) and allowed to undergo extraction using method A or B. The sample extract was syringe filtered and an aliquot (5 mL) was mixed with water (5 mL) prior to SPE, while another aliquot (1 mL) was set aside for HPLC measurements. 0.45 µm syringe filters

from Pall were used for filtering organic sample solutions for voltammetric and HPLC measurements.

Table 6.1: Respective volume of CAPs stock solution make up to 5 mL with electrolyte stock solution to achieve the required CAPs concentration.

CAPs conc. in MeCN/ ppm	Vol. of CAPs stock solution/ μL
70.1	20
140.2	40
210.4	60
280.5	80
350.6	100
525.9	150
701.2	200
1051.8	300
1402.3	400

Oasis HLB solid-phase extraction cartridges (6 cc, 500 mg Sorbent, 60 μm pore size) were used to extract the CAPs in the sample solutions. The 5-step procedure involved conditioning, equilibrating, sample loading, washing and eluting. The cartridge was conditioned with methanol (3 mL) and equilibrated with methanol/water (50:50 %v/v) (5 mL). The 10 mL mixture of the sample extract (5 mL) and water (5 mL) was loaded into the cartridge and subsequently washed with methanol/water (50:50 %v/v) (5 mL). A 5 mL electrolyte solution containing 0.2 M of supporting electrolyte in MeCN with 11.1 M water was used to elute CAPs from the cartridge into a 5 mL volumetric flask. After each step, vacuum was applied to the cartridge to remove any excess solvent in the cartridge. The concentration of CAPs in the sample extract (5 mL) prior to

addition of water was assumed to be the same as the eluted solution containing extracted CAPs, since the initial and final volume were kept constant.

6.1.4. Instrumentation

The mobile phase used for HPLC analysis of CAPs consisted of methanol-water (65:35 %v/v) with a flow rate of 1.0 mL/min and an injection volume of 20 μ L and UV-vis detector set at 280 nm. The chilli samples were centrifuged using Sorvall Lynx 6000 centrifuge, with rotor A23-6 $\times$ 100 in Oak Ridge centrifuge tube with sealing caps (Thermo Scientific).

6.1.5. Voltammetric Experiments

The 5 mL solutions of various concentration of CAPs obtained for calibration or of the various sample solutions for CAPs detection were separated into five 1 mL solutions in 1.5 mL microcentrifuge tube. The CAPs content was separately determined in each of the five solutions using square-wave voltammetry (SWV) and an average of the five measurements was used to calculate the CAPs concentration in the initial sample. The sample solutions were spiked with an aliquot of the CAPs stock solution to confirm the CAPs oxidation peak. SWV experiments were carried out under a step potential of 5 mV and modulation amplitude of 20 mV.

6.2. Results and Discussion

6.2.1. Voltammetry of CAPs in MeCN with Varying Water Content

CAPs have a vanillyl functional group which is comprised of a phenolic moiety. Phenolic compounds are known to undergo hydrogen bonding interactions with trace water in organic solvents,^[3] which shifts the oxidative peak potentials (E_p^{ox}). Results from Chapter 5 showed a shift in the oxidation potentials to less positive potentials with increasing water content as well as a decrease in the peak height. Since the peak height will be used to quantify CAPs, an optimal water content in MeCN have to be determined such that the peak height would not be affected by the water content and could be suitable for use to elute and detect CAPs. Thus, a SWV study of increasing water content with a fixed amount of CAPs in MeCN was performed. A similar trend in which the oxidation potential shifts to lower oxidation potential with increasing water content was observed in SWV as shown in Figure 6.1. The SWV of CAPs without the addition of water gave two peaks at 0.65 V and 0.9 V vs. Fc/Fc^+ (Figure 6.1 (left), Red line).

Figure 6.1: (Left) SWV of fixed amounts of CAPs in MeCN with 0.2 M $n\text{-Bu}_4\text{NPF}_6$ with increasing water content using 1 mm diameter GC electrode. (Blank scan is in black dotted line) Water content: 5.7 mM (no addition of water, Red line), 1.1 M, 2.2 M, 4.4 M, 6.7 M, 8.9 M, 11.1 M, 13.3 M). (Right) Plot of peak height with water content in MeCN.

After the water content was increased to 1 M, only one anodic peak at 0.55 V vs. Fc/Fc^+ was observed. As the water content increased further, the peak shifted to even less positive potential with a decrease in the peak height. A plot of the peak height with the water content in MeCN is shown in Figure 6.1 (Right). The peak height of CAPs was observed to decrease as the water content increased and levelled off at a water content of about 11.1 M. Although the peak height was also observed to drop further when the water content was increased past 11.1 M, the decrease in peak height was considered marginal. As such, MeCN with a water content of 11.1 M was used throughout the later sections for creating the calibration curve as well as for the elution of CAPs during the SPE process. The use of MeCN with a high water content is instrumental in the later analysis to minimise the effect of atmospheric moisture or moisture from sample being introduced into the test solution, as any further increase in the water content will only have minimal effect on the peak height of the CAPs obtained in SWV.

As the capsaicin used in this study is natural grade, it consists of capsaicin, dihydrocapsaicin and small amounts of nordihydrocapsaicin. The structural differences between them lies only in the aliphatic chain with different degrees of unsaturation. A comparison of the electrochemical profiles of capsaicin and nonivamide in Chapter 5 showed no significant differences were observed between the two voltammograms of the two compounds. This suggests that differences in the carbon chain has a negligible influence on the voltammetric behaviour. Thus, the various CAPs, which only differ from each other by the aliphatic chain, can be collectively quantified by the peak height in square-wave voltammetry (SWV).

6.2.2. SWV of CAPs in MeCN with Varying Water Content and Calibration Plot

In order to reduce the amount of the solvent and electrolyte used, the total volume of the solution for SWV measurements was kept at 1 mL using a 1.5 mL microcentrifuge tube.^[1] (See Appendix 8.2) This was also made possible by the use of electrodes which were small and about 2 to 3 mm

in diameter, which allowed them to fit into the microcentrifuge tube easily. Furthermore, microcentrifuge tubes are small and disposable, which makes them portable and easy to use.

To quantify CAPs in MeCN, SWV was also adopted as it can allow the peak height and potentials to be swiftly obtained. SWV of CAPs in the concentration range of 70 to 1400 ppm on GC electrode in MeCN with its corresponding calibration plot of peak height against concentration are plotted in Figure 6.2. The peak potential of CAPs on GC electrode occurs at ca. 0.42 V to 0.51 V vs. Fc/Fc^+ in MeCN. The peak height increased linearly with increasing concentration of CAPs with a high correlation coefficient, R^2 of 0.999. A relative standard deviation of 7 % or lower was obtained for the five replicates of the concentrations between 70 to 1400 ppm, suggesting excellent repeatability. The percentage recoveries for the SPE procedure were found to be between 86.9 % to 89.0 %. The percentage recoveries were calculated using the recovery of eluted CAPs from a known concentration of CAPs loaded into the SPE cartridge.

As SPE will be coupled with SWV for the analysis of CAPs, a larger volume of the sample solution could be passed through the SPE cartridge to enrich the sample if required. However, no sample enrichment was performed in this study.

Figure 6.2: (Left) SWV of increasing concentration of CAPs in MeCN with 11.1 M water and 0.2 M *n*-Bu₄NPF₆ using 1 mm diameter GC electrode (70.1 ppm, 140.2 ppm, 210.4 ppm, 280.5 ppm, 350.6 ppm, 525.9 ppm, 701.2 ppm, 1051.8 ppm and 1402.3 ppm). Black dotted line denotes a blank scan. (Right): Calibration plot of CAPs peak height with increasing concentrations. Standard deviations marked in red for each concentration were obtained from five replicates.

6.2.3. Extraction of CAPs from Samples

Numerous solvents have been reported for the extraction of CAPs from chilli samples.^[2,4] In this study, three commonly used solvents (Ethanol, Methanol and Acetonitrile) and a control solvent (Water) were examined and studied to determine a solvent with the highest CAPs extraction capability. The four solvents above were used to extract commercially available cayenne powder with a high Scoville Heat Unit (SHU) value. The CAPs were extracted using procedure A adopted from Kachoosangi.^[2] It involved sonication of the samples for ten minutes, followed by stirring for two hours and lastly centrifugation at 10,000 rpm for twenty minutes. The supernatant solution was then analysed with HPLC to determine the amount of CAPs extracted. The HPLC of the extracts from the four solvents are provided in Appendix 8.4. CAPs could not be detected when water was used as a solvent, suggesting that CAPs could not be extracted into water. Comparing between the three other solvents, methanol was found to extract slightly more CAPs, than ethanol and acetonitrile. Hence, methanol was the choice of the extraction solvent.

Time consuming extraction procedures of CAFs involving sonication, stirring, centrifugation were employed in some studies.^[2,5] Simplification of the extraction procedure was attempted to reduce processing time. Thus, two extraction procedures A and B were studied. Procedure A involved sonication of the samples for ten minutes, followed by stirring for two hours and lastly centrifugation at 10,000 rpm for twenty minutes, while procedure B required only sonication of the chilli samples for ten minutes. A comparison between the two procedures was made on the extraction of all chilli samples. The chilli samples used in this study were commercially available paprika powder (PAP), cayenne powder (CAY) and tabasco chilli sauce (TAB) as well as self-grinded chilli seed (SEED) and finely cut chilli skin (SKIN) from the supermarket. They were chosen to incorporate both solid and liquid samples with low and high SHU values.

6.2.4. SWV of Post-SPE Sample Solution in MeCN

To quantify CAPs present in the chilli samples, extracts of the chilli samples were treated by SPE to change the solvent medium from methanol to acetonitrile, while retaining CAPs in the sample extracts and removing water-soluble interferences present in the extract. The SPE procedure used involved five steps - conditioning of the extraction cartridge with methanol, equilibrating of the cartridge with methanol/water (50:50 %v/v), loading of premixed sample extract in methanol with water (50:50 %v/v), washing of cartridge with methanol/water (50:50 %v/v) and eluting of CAPs with MeCN containing 11.1 M water premixed with 0.2 M supporting electrolyte. The solvent mixture of methanol/water (50:50 %v/v) was found to have a polarity that would not elute CAPs during the whole SPE process and was a convenient solvent composition to prepare. Hence, it was used in the equilibrating, loading and washing steps. Premixing of the supporting electrolyte into MeCN for elution further simplified the elution process and making it convenient. CAPs were not detected in the eluate during sample loading and washing with methanol/water (50:50 %v/v), confirming that the CAPs were retained in the SPE cartridge. (see Appendix 8.4.)

SWVs of the SPE sample extracts obtained from procedure A on GC electrode in MeCN containing the supporting electrolyte are as shown in Figure 6.3. It was observed that the anodic

peak potential of CAPs in the SPE extracted samples lie between 0.45 V and 0.55 V vs. Fc/Fc^+ . Although the range at which the anodic peak potential appeared lies slightly out of the range obtained from calibration, spiking of the SPE samples with CAPs standard solution showed an increase in the peak height corresponding to CAPs (Figure 6.3, Dotted line). This suggests that the peak potential between 0.45 V and 0.55 V vs. Fc/Fc^+ is indicative of CAPs.

The CAPs content of the SPE sample solutions that were subjected to both procedures A and B were determined using the SWV calibration plot above and compared with the results obtained from HPLC measurements and are tabulated in Table 6.2.

Figure 6.3: (a) Cayenne Pepper powder, (b) Paprika powder, (c) Tabasco Pepper Sauce, (d) Red Chilli Seed, (e) Red Chilli Skin. SWV of post-SPE sample solution (Solid line, —) and after spiking with CAPs stock solution (Dotted line,) in MeCN with 11.1 M water and 0.2 M $n\text{-Bu}_4\text{NPF}_6$ using 1 mm diameter GC electrode.

The CAPs content of the SPE sample solutions obtained via voltammetric means were generally in good agreement (within 34 %) of the values obtained by HPLC, with the largest difference between the two methods for the tabasco and chilli skin extracts. The low CAPs content in the tabasco extract of 17 ppm measured by SWV is lower than the detection limit of 41.3 ppm. Therefore, this explains the relatively poor agreement between the voltammetric measurements and the HPLC measurements of the tabasco extract. The SWV of the chilli skin extract showed

another peak around 0.1 V vs. Fc/Fc^+ . This additional peak might have interfered with the peak height selection of CAPs, resulting in a lower reported CAPs content compared to HPLC measurements. Although SPE should have reduced a significant amount of interferences in the sample extract, some organic matter might have been eluted together with CAPs when MeCN was passed through the cartridge, explaining the presence of the extra peak. It was found that the extract from paprika (which has a low Scoville Heat Unit range) did not display a peak in the region corresponding to CAPs.

The CAPs content of tabasco and cayenne pepper measured via SWV and converted to Scoville Heat Unit scale (assuming 1 ppm = 16 SHU) ^[6] of 2107 and 53,635 were in close agreement to the expected Scoville Heat Unit range of 2500 - 5000 and 30,000 - 50,000 respectively. While the chilli seed and skin that was used in this experiment resembled the Bird's Eye Chilli, the combined and converted Scoville Heat Unit (from HPLC) of the chilli seed and skin of 34,900 SHU was significantly below the expected Scoville Heat Unit range of 100,000 - 225,000 for Bird's Eye Chilli. This might be attributed to the sample preparation as fresh red chilli was used. Some of the fluid in the chilli, which might also contain CAPs, was lost during the grinding of the chilli seed and cutting of the chilli skin. Despite the disparity of the SHU value of the red chilli from the expected value, the CAPs content obtained through SWV was in agreement of the HPLC measurements.

When comparing the CAPs content in the sample using procedure A and B, and taking into account the different sample mass, it was found that procedure A generally extracted more CAPs than procedure B, although the differences were usually very small. This is expected as the duration of sample preparation for procedure A is longer than B. Most samples were either in powder or liquid forms, so it is straightforward to mix or shake them to ensure homogeneity in the sample. However, it is difficult to achieve homogeneity for chilli skin even though it was cut into smaller pieces from several chillies and mixed together. Thus, it might explain the difference in trends for chilli skin, where procedure B resulted in higher values than procedure A. Nonetheless, from the results in Table 6.2, it can be seen that procedure B, which is not as time-consuming,

often does give results comparable to procedure A. Therefore, using the less time-consuming procedure B as well as methanol as an extraction solvent could suffice for sample analysis in many cases.

Table 6.2: Determination of CAPs in samples extracted under procedures A and B using voltammetric methods in MeCN ^[a] compared to HPLC.

Proce- dure	Sample	Sample Mass/g	Conc. in extract/ ppm	Conc. in sample/ ppm		Conc. in sample/ SHU ^[b]		Reference Scoville Heat Unit
			SWV	SWV	HPLC	SWV	HPLC	
A	PAP	1.00119	N.D. ^[c]	N.D. ^[c]	N.D. ^[c]	N.D. ^[c]	N.D. ^[c]	0 - 300
	TAB	1.00552	16.8 (± 3)	131.7	192.8	2107	3085	2500 - 5000
	CAY	1.00183	427.3 (± 1)	3352.2	3816.9	53,635	61,071	30,000 - 50,000
	SEED	0.81103	70.4 (± 1)	682.6	768.3	10,922	12,293	
	SKIN	1.00126	137.6 (± 1)	1080.5	1412.9	17,288	22,606	
B	PAP	1.00613	N.D. ^[c]	N.D. ^[c]	N.D. ^[c]	N.D. ^[c]	N.D. ^[c]	0 - 300
	TAB	1.00292	16.4 (± 1)	128.7	194.9	2059	3119	2500 - 5000
	CAY	1.00289	376.7 (± 1)	2952.3	3302.0	47,238	52,832	30,000 - 50,000
	SEED	0.81590	66.2 (± 1)	637.3	704.1	10,197	11,265	
	SKIN	1.07684	160.2 (± 1)	1169.6	1546.0	18,714	24,735	

[a] Five replicate measurements were made on each sample solution. Values in paranthesis are % RSD.

[b] 1 ppm was assumed to be 16 SHU. ^[6]

[c] N.D.: Not detected.

6.3. Conclusion

Methanol was found to be the best solvent for extracting CAPs compared to ethanol and acetonitrile. The extracted CAPs were then successfully transferred from methanol into acetonitrile for voltammetric analysis using SPE. The CAPs content obtained using the voltammetric method were in line with results obtained from HPLC. The quantity of CAPs extracted with only ten minutes of sonication (procedure B) gave results comparable to another more time-consuming procedure (A) that involved sonication, stirring and centrifugation. The use of SPE prior to voltammetric detection can reduce water-soluble compounds which might interfere with the detection of CAPs and also allows for preconcentration of samples with low levels of CAPs.

6.4. References

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Conclusion

Food molecules undergo oxidation reactions in the body, which could result in the bioactivity observed. Knowledge of the intermediates formed is often implied from the isolation of the products. Using an electrochemical approach, the compounds can be studied in a more simplified and controlled manner and useful information on the intermediates can potentially be obtained. Using their electrochemical behaviour in organic solvents, these compounds can also be quantified electrochemically after they have been extracted into organic solvents.

In this thesis, electrochemical studies were performed on two molecules present in food and beverages widely consumed worldwide, caffeine and capsaicin, to examine the oxidation mechanism in the aprotic organic solvent acetonitrile on Pt and GC electrodes. Based on the electrochemical behaviour observed, electrochemical detection methods were developed to quantify the compounds in MeCN.

Caffeine (CAF) undergoes a one-electron oxidation in MeCN at 1.2 V vs. Fc/Fc^+ on Pt electrode to form a cation radical. The lifetime of the cation radical improved as the trace water content in the solvent is decreased. Electrochemical oxidation (and chemical oxidation with NOSbF_6) of CAF in MeCN leads to the generation of the protonated CAF cation as the long-term product in high yield, whose structure was confirmed by single-crystal X-ray crystallography and NMR spectroscopy. The protonated cation is able to be electrochemically reduced back to CAF under electrolysis conditions. The formation of the protonated cation involves the initial one-electron oxidation of CAF to form the cation radical, which undergoes a hydrogen atom abstraction reaction to afford the protonated cation. Digital simulations of the CV data show that the rate and equilibrium constants for the hydrogen atom abstraction mechanism are $k_f = 1.0 \times 10^2 \text{ L mol}^{-1} \text{ s}^{-1}$ and $K_{\text{eq}} = 1.0 \times 10^2$.

Voltammetric quantification of CAF in MeCN was performed after aqueous sample solutions were extracted into MeCN using SPE. The voltammetric detection of CAF in MeCN after SPE could be performed in small amounts such as in a microcentrifuge tube in order to minimise the required amount of MeCN and reagents used during the analysis. The voltammetric determination of CAF from a range of beverages extracted into MeCN was in good agreement with the results obtained from HPLC measurements on the same samples. The SPE-coupled voltammetric method also gave more accurate results than those obtained via the direct voltammetric detection of CAF in real beverage samples on bare electrodes.

Capsaicin (CAPH) gave two chemically irreversible anodic peaks at ca. 0.7 V and 1.0 V vs. Fc/Fc^+ and two cathodic peaks at ca. 0.1 V and -0.4 V vs. Fc/Fc^+ on GC electrode when the scan direction was reversed under dried conditions. Under wet conditions, only one chemically

irreversible anodic peak was observed at ca. 0.5 V vs. Fc/Fc^+ with one cathodic peak at ca. 0 V vs. Fc/Fc^+ upon reversal of the scan direction. A total of two electrons being transferred during the oxidation process (under dried and wet conditions) was confirmed by combining the CPE results in wet MeCN as well as looking at the peak height of the anodic peak under dried and wet conditions. A hydrogen bonding interaction between the phenolic oxygen atom and the protons on the 2-methoxy group was invoked in order to explain the two anodic peaks each consisting of the transfer of one electron observed under dried conditions. The cathodic peak at 0 V vs. Fc/Fc^+ that was observed when the scan direction was reversed under wet conditions was outrun at higher scan rates suggesting the reduction of an *o*-quinone intermediate formed after hydrolysis reaction of the initially formed phenoxonium cation CAP^+ with water. The cathodic peak at -0.4 V vs. Fc/Fc^+ was assigned to the reduction of a dimeric product previously reported and observed in a controlled-potential electrolysis experiment of nonivamide in a mixture of water and acetonitrile containing ammonium formate.

Voltammetric quantification of capsaicinoids (CAPs) in MeCN was performed following a similar approach as that with CAF. CAPs from a range of spices and chillies were extracted using methanol prior to further extraction into MeCN using SPE. Based on the electrochemical behaviour, a solution of MeCN with water content of 11.1 M was used as the eluent. Voltammetric detection of CAPs extracted from various chilli samples into MeCN was in very good agreement with quantification using HPLC. The extraction procedure using only sonication gave results comparable to a procedure using a combination of sonication, stirring and centrifuging.

The electrochemical studies of CAF and CAPH in MeCN showed that the water content plays a role in the formation of intermediates produced during oxidation. Lower water content in the solvent can reduce chemical reactions, which would otherwise result in the formation of other products. Quantification of CAF and CAPs in MeCN indicates that voltammetric detection of electroactive molecules is not only limited to aqueous solutions with electrode modification and

pretreatment but has been shown to be possible after the molecule of interest has been extracted by SPE before detection.

8

Appendix

Appendix 8.1. Supporting Information for Chapter 3

Appendix 8.2. Supporting Information for Chapter 4

Appendix 8.3. Supporting Information for Chapter 5

Appendix 8.4. Supporting Information for Chapter 6

Appendix 8.1.
Supporting Information for Chapter 3

Figure A1: (Left) Current vs. time data obtained during the exhaustive oxidation of CAF in initially dried MeCN containing 0.2 M $n\text{-Bu}_4\text{NPF}_6$ at 1.95 V vs. Ag wire. (Right) No. of electrons transferred during CPE.

Figure A2: (Left) Current vs. time data obtained during the exhaustive reduction of the oxidised product of CAF in MeCN containing 0.2 M $n\text{-Bu}_4\text{NPF}_6$ at 0 V vs. Ag wire. (Right) No. of electrons transferred during CPE.

Figure A3: ^1H NMR spectrum of caffeine in CD_3CN .

Figure A4: ^{13}C NMR spectrum of caffeine in CD_3CN .

Figure A5: ^1H NMR spectrum of protonated caffeine hexafluoroantimonate dihydrate in CD_3CN .

Figure A6: ^{13}C NMR spectrum of protonated caffeine hexafluoroantimonate dihydrate in CD_3CN .

Figure A7: HMQC NMR spectrum of protonated caffeine hexafluoroantimonate dihydrate in CD_3CN .

Crystal Structure Report for Protonated Caffeine Hexafluoroantimonate Dihydrate

A colourless plate-like specimen of $\text{C}_8\text{H}_{15}\text{F}_6\text{N}_4\text{O}_4\text{Sb}$, approximate dimensions 0.060 mm $\times$ 0.120 mm $\times$ 0.300 mm, was used for the X-ray crystallographic analysis. The X-ray intensity data were measured.

A total of 700 frames were collected. The total exposure time was 1.17 hours. The frames were integrated with the Bruker SAINT software package using a narrow-frame algorithm. The integration of the data using a monoclinic unit cell yielded a total of 3778 reflections to a maximum θ angle of 28.30° (0.75 Å resolution), of which 3778 were independent (average redundancy 1.000, completeness = 99.9 %) and 3498 (92.59 %) were greater than $2\sigma(F^2)$. The final cell constants of $a = 7.6526(5)$ Å, $b = 12.1976(8)$ Å, $c = 16.0911(11)$ Å, $\beta = 95.340(2)^\circ$, volume = $1495.48(17)$ Å³, are based upon the refinement of the XYZ-centroids of 5580 reflections above $20 \sigma(I)$ with $5.085^\circ < 2\theta < 56.09^\circ$. Data were corrected for absorption effects using the multi-scan method (SADABS). The ratio of minimum to maximum apparent transmission was 0.801. The calculated minimum and maximum transmission coefficients (based on crystal size) are 0.5940 and 0.8930.

The final anisotropic full-matrix least-squares refinement on F^2 with 212 variables converged at $R1 = 3.28$ %, for the observed data and $wR2 = 7.95$ % for all data. The goodness-of-fit was 1.071. The largest peak in the final difference electron density synthesis was $1.215 \text{ e}^-/\text{\AA}^3$ and the largest hole was $-1.360 \text{ e}^-/\text{\AA}^3$ with an RMS deviation of $0.143 \text{ e}^-/\text{\AA}^3$. On the basis of the final model, the calculated density was 2.074 g/cm^3 and $F(000)$, 912 e^- .

Figure A8: ORTEP drawing of protonated caffeine hexafluoroantimonate dihydrate with thermal ellipsoids at 50% probability levels.

Table A1: Sample and Crystal data for protonated caffeine hexafluoroantimonate dihydrate.

Identification code	Protonated Caffeine Hexafluoroantimonate Dihydrate	
Chemical formula	$\text{C}_8\text{H}_{15}\text{F}_6\text{N}_4\text{O}_4\text{Sb}$	
Formula weight	466.99	
Temperature	103(2) K	
Wavelength	0.71073 Å	
Crystal size	0.060 x 0.120 x 0.300 mm	
Crystal habit	Colourless plate	
Crystal system	Monoclinic	
Space group	P 1 21/n 1	
Unit cell dimensions	a = 7.6526(5) Å	$\alpha = 90^\circ$
	b = 12.1976(8) Å	$\beta = 95.340(2)^\circ$
	c = 16.0911(11) Å	$\gamma = 90^\circ$
Volume	1495.48(17) Å ³	
Z	4	
Density (calculated)	2.074 g/cm ³	
Absorption coefficient	1.936 mm ⁻¹	
F(000)	912	

Table A2: Data collection and structure refinement for protonated caffeine hexafluoroantimonate dihydrate.

Theta range for data collection	2.85 to 28.30°	
Reflections collected	3778	
Coverage of independent reflections	99.9%	
Absorption correction	multi-scan	
Max. and min. transmission	0.8930 and 0.5940	
Refinement method	Full-matrix least-squares on F^2	
Refinement program	SHELXL-2013 (Sheldrick, 2013)	
Function minimized	$\Sigma w(F_o^2 - F_c^2)^2$	
Data / restraints / parameters	3778 / 0 / 212	
Goodness-of-fit on F^2	1.071	
$\Delta/\sigma_{\max}$	0.001	
Final R indices	3498 data; $I > 2\sigma(I)$	R1 = 0.0328, wR2 = 0.0770
	all data	R1 = 0.0373, wR2 = 0.0795
Weighting scheme	$w = 1/[\sigma^2(F_o^2) + (0.0266P)^2 + 2.3694P]$ where $P = (F_o^2 + 2F_c^2)/3$	
Largest diff. peak and hole	1.215 and -1.360 eÅ ⁻³	
R.M.S. deviation from mean	0.143 eÅ ⁻³	

Table A3: Atomic coordinates and equivalent isotropic atomic displacement parameters ($\text{\AA}^2$) for protonated caffeine hexafluoroantimonate dihydrate.

$U(\text{eq})$ is defined as one third of the trace of the orthogonalized U_{ij} tensor.

	x/a	y/b	z/c	U(eq)
Sb1	0.50092(4)	0.78183(2)	0.75974(2)	0.01304(8)
C1	0.6444(6)	0.0778(3)	0.9105(3)	0.0211(9)
C2	0.5923(5)	0.2754(3)	0.8863(2)	0.0148(7)
C3	0.5355(6)	0.4666(3)	0.8562(2)	0.0186(8)
C4	0.7288(5)	0.4182(3)	0.9823(2)	0.0144(8)
C5	0.8050(5)	0.3280(3)	0.0288(2)	0.0122(7)
C6	0.7749(5)	0.2220(3)	0.0055(2)	0.0128(7)
C7	0.9424(5)	0.2203(4)	0.1224(2)	0.0159(8)
C8	0.9768(6)	0.4192(3)	0.1533(2)	0.0174(7)
F1	0.6729(3)	0.6728(2)	0.74933(16)	0.0232(6)
F2	0.5046(4)	0.7490(2)	0.87543(14)	0.0257(6)
F3	0.3371(3)	0.8939(2)	0.77458(15)	0.0211(5)
F4	0.5114(3)	0.8231(2)	0.64697(14)	0.0235(6)
F5	0.3243(4)	0.6743(2)	0.73332(19)	0.0352(7)
F6	0.6844(3)	0.8820(2)	0.78829(16)	0.0250(6)
N1	0.6700(4)	0.1927(3)	0.93595(19)	0.0136(7)
N2	0.6205(5)	0.3827(3)	0.91198(19)	0.0138(6)
N3	0.9118(4)	0.3242(3)	0.10349(19)	0.0131(7)
N4	0.8615(5)	0.1557(3)	0.06353(18)	0.0144(6)
O1	0.5009(4)	0.2534(2)	0.82202(16)	0.0185(6)
O2	0.7509(4)	0.5154(2)	0.99872(16)	0.0184(6)
O3	0.8850(5)	0.9412(2)	0.08630(18)	0.0291(7)
O4	0.7629(4)	0.7573(2)	0.00735(18)	0.0299(8)

Table A4: Bond lengths (Å) for protonated caffeine hexafluoroantimonate dihydrate.

Sb1-F3	1.885(2)	Sb1-F6	1.885(3)
Sb1-F1	1.889(2)	Sb1-F4	1.892(2)
Sb1-F2	1.902(2)	Sb1-F5	1.903(3)
C1-N1	1.468(5)	C1-H1A	0.98
C1-H1B	0.98	C1-H1C	0.98
C2-O1	1.224(5)	C2-N2	1.383(5)
C2-N1	1.386(5)	C3-N2	1.473(5)
C3-H3A	0.98	C3-H3B	0.98
C3-H3C	0.98	C4-O2	1.223(5)
C4-N2	1.407(5)	C4-C5	1.425(5)
C5-C6	1.360(5)	C5-N3	1.390(5)
C6-N4	1.361(5)	C6-N1	1.362(5)
C7-N3	1.319(5)	C7-N4	1.340(5)
C7-H7	0.95	C8-N3	1.469(5)
C8-H8A	0.98	C8-H8B	0.98
C8-H8C	0.98	N4-H4	0.88
O3-H1O	0.8433	O3-H2O	0.85
O4-H3O	0.8321	O4-H4O	0.8378

Table A5: Bond angles (°) for protonated caffeine hexafluoroantimonate dihydrate.

F3-Sb1-F6	89.32(11)	F3-Sb1-F1	177.04(11)
F6-Sb1-F1	88.21(11)	F3-Sb1-F4	90.96(11)
F6-Sb1-F4	87.97(11)	F1-Sb1-F4	90.58(11)
F3-Sb1-F2	88.74(11)	F6-Sb1-F2	87.41(11)
F1-Sb1-F2	89.52(12)	F4-Sb1-F2	175.37(11)
F3-Sb1-F5	93.51(12)	F6-Sb1-F5	176.77(12)
F1-Sb1-F5	88.91(12)	F4-Sb1-F5	93.52(12)
F2-Sb1-F5	91.11(12)	N1-C1-H1A	109.5
N1-C1-H1B	109.5	H1A-C1-H1B	109.5
N1-C1-H1C	109.5	H1A-C1-H1C	109.5
H1B-C1-H1C	109.5	O1-C2-N2	121.4(4)
O1-C2-N1	120.6(4)	N2-C2-N1	118.1(3)
N2-C3-H3A	109.5	N2-C3-H3B	109.5
H3A-C3-H3B	109.5	N2-C3-H3C	109.5
H3A-C3-H3C	109.5	H3B-C3-H3C	109.5
O2-C4-N2	122.1(4)	O2-C4-C5	126.3(4)
N2-C4-C5	111.5(3)	C6-C5-N3	106.1(3)
C6-C5-C4	122.6(3)	N3-C5-C4	131.3(4)
C5-C6-N4	108.4(3)	C5-C6-N1	123.2(4)
N4-C6-N1	128.3(4)	N3-C7-N4	110.0(3)
N3-C7-H7	125.0	N4-C7-H7	125.0
N3-C8-H8A	109.5	N3-C8-H8B	109.5
H8A-C8-H8B	109.5	N3-C8-H8C	109.5
H8A-C8-H8C	109.5	H8B-C8-H8C	109.5
C6-N1-C2	118.1(3)	C6-N1-C1	122.2(3)
C2-N1-C1	119.7(3)	C2-N2-C4	126.5(3)
C2-N2-C3	115.3(3)	C4-N2-C3	118.0(3)
C7-N3-C5	108.0(3)	C7-N3-C8	126.0(3)
C5-N3-C8	125.9(3)	C7-N4-C6	107.4(3)
C7-N4-H4	126.3	C6-N4-H4	126.3
H1O-O3-H2O	111.6	H3O-O4-H4O	114.9

Table A6: Anisotropic atomic displacement parameters ($\text{\AA}^2$) for protonated caffeine hexafluoroantimonate dihydrate.

The anisotropic atomic displacement factor exponent takes the form: $-2\pi^2 [h^2 a^{*2} U_{11} + \dots + 2 h k a^* b^* U_{12}]$

	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
Sb1	0.01342(12)	0.01307(12)	0.01289(12)	0.00096(10)	0.00254(12)	0.00013(11)
C1	0.028(2)	0.0145(19)	0.020(2)	-0.0016(15)	-0.0030(18)	-0.0033(18)
C2	0.0131(16)	0.019(2)	0.0128(16)	0.0004(15)	0.0051(14)	-0.0015(16)
C3	0.021(2)	0.0186(19)	0.0159(18)	0.0051(14)	0.0006(17)	0.0053(18)
C4	0.0124(18)	0.020(2)	0.0114(16)	0.0011(14)	0.0035(14)	0.0016(16)
C5	0.0125(18)	0.0164(19)	0.0081(16)	0.0005(14)	0.0032(13)	-0.0001(15)
C6	0.0138(17)	0.0133(17)	0.0113(15)	0.0002(15)	0.0022(13)	0.0004(17)
C7	0.017(2)	0.0185(18)	0.0124(16)	0.0032(16)	0.0019(13)	0.0035(18)
C8	0.022(2)	0.0152(18)	0.0146(17)	-0.0007(14)	-0.0003(17)	-0.0022(19)
F1	0.0146(12)	0.0194(13)	0.0362(14)	0.0022(11)	0.0059(11)	0.0054(10)
F2	0.0271(14)	0.0374(15)	0.0123(11)	0.0056(9)	0.0005(10)	0.0002(12)
F3	0.0228(12)	0.0178(13)	0.0231(12)	-0.0001(10)	0.0050(11)	0.0048(11)
F4	0.0306(15)	0.0264(13)	0.0143(11)	0.0006(9)	0.0057(10)	0.0010(11)
F5	0.0353(16)	0.0268(15)	0.0443(17)	-0.0014(13)	0.0083(15)	-0.0043(13)
F6	0.0225(13)	0.0243(14)	0.0282(13)	-0.0060(11)	0.0025(11)	-0.0051(12)
N1	0.0178(18)	0.0135(17)	0.0090(14)	-0.0013(11)	-0.0014(12)	-0.0016(12)
N2	0.0168(16)	0.0145(15)	0.0103(14)	0.0012(12)	0.0012(14)	0.0013(15)
N3	0.0145(16)	0.0142(15)	0.0099(14)	-0.0010(12)	-0.0020(12)	-0.0018(12)
N4	0.0208(16)	0.0123(15)	0.0103(14)	0.0005(11)	0.0021(14)	0.0019(15)
O1	0.0190(14)	0.0230(15)	0.0128(12)	-0.0006(10)	-0.0020(12)	-0.0008(13)
O2	0.0244(17)	0.0136(15)	0.0169(13)	-0.0007(11)	0.0012(12)	0.0018(13)
O3	0.054(2)	0.0149(15)	0.0171(14)	0.0015(11)	-0.0021(16)	-0.0007(16)
O4	0.039(2)	0.0213(18)	0.0268(16)	-0.0021(13)	-0.0108(16)	0.0039(16)

Table A7: Hydrogen atomic coordinates and isotropic atomic displacement parameters ($\text{\AA}^2$) for protonated caffeine hexafluoroantimonate dihydrate.

	x/a	y/b	z/c	U(eq)
H1A	0.7206	0.0605	0.8666	0.032
H1B	0.5216	0.0662	0.8893	0.032
H1C	0.6740	0.0299	0.9587	0.032
H3A	0.5933	0.4693	0.8044	0.028
H3B	0.5453	0.5383	0.8837	0.028
H3C	0.4113	0.4481	0.8433	0.028
H7	1.0116	0.1951	1.1707	0.019
H8A	0.8786	0.4550	1.1774	0.026
H8B	1.0321	0.4713	1.1174	0.026
H8C	1.0632	0.3948	1.1982	0.026
H4	0.8639	0.0836	1.0626	0.017
H1O	0.8909	0.9209	0.1366	0.044
H2O	0.8633	0.8878	0.0530	0.044
H3O	0.8374	0.7082	1.0040	0.045
H4O	0.6752	0.7529	0.9722	0.045

Appendix 8.2.

Supporting Information for Chapter 4

Figure B1: (Top to Bottom) HPLC of increasing concentrations of caffeine in water (0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM and 1 mM) using methanol-phosphate buffer pH 3 (30:70 %v/v) mobile phase with a flow rate of 1.0 mL/min, injection volume of 20 μ L and UV-vis detector set at 268 nm.

Figure B2: Calibration plot of HPLC peak height with increasing concentrations of caffeine in water (0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM and 1 mM) using methanol-phosphate buffer pH 3 (30:70 %v/v) mobile phase.

Figure B3: Set up for voltammetric detection in microcentrifuge tube.

Figure B4: (Top to Bottom) HPLC of different beverages (Coca-Cola, RedBull, Sprite, Pokka Green Tea, Moccona Coffee, Moccona Coffee with Creamer, Moccona Decaffeinated Coffee and Lipton Tea) using methanol-phosphate buffer pH 3 (30:70 %v/v) mobile phase with a flow rate of 1.0 mL/min, injection volume of 20 μ L and UV-vis detector set at 268 nm.

Figure B5: (Top to Bottom) HPLC of the eluate of the different beverage after passing through solid-phase extraction cartridge (Coca-Cola, RedBull, Sprite, Pokka Green Tea, Moccona Coffee, Moccona Coffee with Creamer, Moccona Decaffeinated Coffee and Lipton Tea) using methanol-phosphate buffer pH 3 (30:70 %v/v) mobile phase with a flow rate of 1.0 mL/min, injection volume of 20 μ L and UV-vis detector set at 268 nm.

Figure B6: (Top to Bottom) HPLC of increasing concentrations of caffeine in water (0.2 mM, 0.4 mM, 0.6 mM) using methanol-phosphate buffer pH 3 (20:80 %v/v) mobile phase with a flow rate of 1.0 mL/min, injection volume of 20 μ L and UV-vis detector set at 268 nm.

Figure B7: Calibration plot of HPLC peak height with increasing concentrations of caffeine in water (0.2 mM, 0.4 mM and 0.6 mM) using methanol-phosphate buffer pH 3 (20:80 %v/v) mobile phase.

Figure B8: (Top to Bottom) HPLC of different beverages (Pokka Green Tea and Moccona Decaffeinated Coffee) using methanol-phosphate buffer pH 3 (20:80 %v/v) mobile phase with a flow rate of 1.0 mL/min, injection volume of 20 μ L and UV-vis detector set at 268 nm.

Appendix 8.3.

Supporting Information for Chapter 5

Figure C1: (Left) Current vs. time data obtained during the exhaustive oxidation of CAPH in initially dried MeCN containing 0.2 M $n\text{-Bu}_4\text{NPF}_6$ at 0.8 V vs. Fc/Fc^+ . (Right) No. of electrons transferred during CPE.

Figure C2: (Left) Current vs. time data obtained during the follow-up exhaustive oxidation of CAPH in MeCN containing 0.2 M $n\text{-Bu}_4\text{NPF}_6$ at 1.1 V vs. Fc/Fc^+ . (Right) No. of electrons transferred during CPE.

Figure C3: (Left) Current vs. time data obtained during the exhaustive oxidation of CAPH in wet MeCN (1 M water) containing 0.2 M $n\text{-Bu}_4\text{NPF}_6$ at 0.8 V vs. Fc/Fc^+ . (Right) No. of electrons transferred during CPE.

Figure C4: (Left) Current vs. time data obtained during the exhaustive oxidation of nonivamide in a mixture of water (4.5 M) and MeCN containing ammonium formate (20 mM) at 0.8 V vs. Ag wire. (Right) No. of electrons transferred during CPE.

Figure C5: Mass spectrum of 2 mM nonivamide in a mixture of water (4.5 M) and acetonitrile containing 20 mM ammonium formate.

Figure C6: Mass spectrum of solution after electrochemical oxidation of 2 mM nonivamide in a mixture of water (4.5 M) and acetonitrile containing 20 mM ammonium formate.

Appendix 8.4.

Supporting Information for Chapter 6

Figure D1: (Top to Bottom) HPLC of increasing concentrations of capsaicinoids in methanol (0 ppm, 99.9 ppm, 199.7 ppm, 299.6 ppm, 399.5 ppm, 499.4 ppm) using methanol-water (65:35 %v/v) mobile phase with a flow rate of 1.0 mL/min, injection volume of 20 μ L and UV-vis detector set at 280 nm.

Figure D2: Calibration plot of HPLC peak height with increasing concentrations of capsaicinoids in methanol (99.9 ppm, 199.7 ppm, 299.6 ppm, 399.5 ppm, 499.4 ppm) using methanol-water (65:35 %v/v) mobile phase.

Figure D3: (Top to Bottom) HPLC of different solvent extracts of Cayenne Pepper Ground Powder (water, acetonitrile, ethanol, methanol) using methanol-water (65:35 %v/v) mobile phase with a flow rate of 1.0 mL/min, injection volume of 20 μ L and UV-vis detector set at 280 nm. Chromatograms were expanded to ensure clarity of the peaks of interest.

Figure D4: Pound and Ground chilli seeds and finely cut chilli skin.

Figure D5: (Top to Bottom) HPLC of methanol extracts of various chilli samples (Paprika Ground Powder, Tabasco Pepper Sauce, Cayenne Pepper Ground Powder, chilli seed, chilli skin) using Procedure A using methanol-water (65:35 %v/v) mobile phase with a flow rate of 1.0 mL/min, injection volume of 20 μ L and UV-vis detector set at 280 nm. Chromatograms were expanded to ensure clarity of the peaks of interest.

Figure D6: (Top to Bottom) HPLC of methanol extracts of various chilli samples (Paprika Ground Powder, Tabasco Pepper Sauce, Cayenne Pepper Ground Powder, chilli seed, chilli skin) using Procedure B using methanol-water (65:35 %v/v) mobile phase with a flow rate of 1.0 mL/min, injection volume of 20 μ L and UV-vis detector set at 280 nm. Chromatograms were expanded to ensure clarity of the peaks of interest.

Figure D7: (Top to Bottom) HPLC of the eluate of methanol extracts of various chilli samples (Paprika Ground Powder, Tabasco Pepper Sauce, Cayenne Pepper Ground Powder, chilli seed, chilli skin) using Procedure A after loading into solid-phase extraction cartridge using methanol-water (65:35 %v/v) mobile phase with a flow rate of 1.0 mL/min, injection volume of 20 μ L and UV-vis detector set at 280 nm. Chromatograms were expanded to ensure clarity of the peaks of interest.

Figure D8: (Top to Bottom) HPLC of the eluate of methanol extracts of various chilli samples (Paprika Ground Powder, Tabasco Pepper Sauce, Cayenne Pepper Ground Powder, chilli seed, chilli skin) using Procedure B after loading into solid-phase extraction cartridge using methanol-water (65:35 %v/v) mobile phase with a flow rate of 1.0 mL/min, injection volume of 20 μ L and UV-vis detector set at 280 nm. Chromatograms were expanded to ensure clarity of the peaks of interest.

Figure D9: (Top to Bottom) HPLC of the eluate of methanol extracts of various chilli samples (Paprika Ground Powder, Tabasco Pepper Sauce, Cayenne Pepper Ground Powder, chilli seed, chilli skin) using Procedure A after washing through solid-phase extraction cartridge using methanol-water (65:35 %v/v) mobile phase with a flow rate of 1.0 mL/min, injection volume of 20 μ L and UV-vis detector set at 280 nm. Chromatograms were expanded to ensure clarity of the peaks of interest.

Figure D10: (Top to Bottom) HPLC of the eluate of methanol extracts of various chilli samples (Paprika Ground Powder, Tabasco Pepper Sauce, Cayenne Pepper Ground Powder, chilli seed, chilli skin) using Procedure B after washing through solid-phase extraction cartridge using methanol-water (65:35 %v/v) mobile phase with a flow rate of 1.0 mL/min, injection volume of 20 μ L and UV-vis detector set at 280 nm. Chromatograms were expanded to ensure clarity of the peaks of interest.