Comparing predefined derivatisation parameters for GC-MS analysis of selected organic acids

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ABSTRACT

Organic acids are intermediates of numerous biochemical pathways in living organisms. They can be detected in biological fluids which make it possible to obtain a representative profile of the functioning of biochemical pathways. General analysis of these acids is based on their distinguishable chemical properties. Chemically, organic acids refer to water-soluble compounds with one or more carboxylic group and often consist of other functional groups. The analysis of organic acids is commonly used for the diagnosis of inborn errors of metabolism, by using gas chromatography-mass spectrometry (GC-MS), which can be seen as the gold standard method. For GC-MS analysis of organic acids, derivatisation prior to analysis is necessary to improve the volatility, separation, selectivity and thermal stability of the compounds. Silylation is a common derivatisation method for organic acids, however disadvantages such as long incubation periods at high temperatures, lack of repeatability and the formation of multiple derivatives that result in multiple peaks on a chromatogram often occur. Therefore, research into the optimum silylation reaction conditions for organic acids are needed. The aim of this investigation was to compare the outcome of predefined silylation derivatisation parameters required for thermal- and microwave-assisted derivatisation, as a prerequisite for GC-MS analysis of selected organic acids. The organic acids were selected through the consideration of their functional groups and physiological concentration to ensure a representative group of the broad organic acid class.

Guided by literature, the silylation conditions included: temperatures (50, 60, 70 and 85°C) in combination with reaction times (30, 60, 90 and 120 min) and microwave energies (150, 230, 350 and 450 W) in combination with reaction times (1.5, 2.0, 3.0 and 4.0 min). Organic acid standards were derivatised and analysed using GC-MS in single ion monitoring mode. Data were processed, and statistical comparisons were performed. Coefficient of variance (CV) was used as the main performance criterion in this study and used as a mean to compare the results. Between conventional thermal- and microwave-assisted derivatisation, conventional thermal derivatisation was found to provide lower variation and to be more robust. Further it was found that the use of methoxymation was beneficial for repeatability of some organic acids, making adapted thermal derivatisation the preferred type. Individual organic acids performed differently at temperature, microwave energy and reaction time increments with inconsistent pattern towards higher/lower temperature or microwave energy and longer/shorter reaction time. Derivatisation efficiency was found to be largely influenced by the structure of the compound, with better repeatability when derivatising only carboxyl groups. From all conditions investigated, 60°C for 30 min was identified as the condition within adapted thermal derivatisation to provide the least variation for the included organic acids. For thermal derivatisation the condition at 70°C for 90 min gave the lowest CV values. Microwave-assisted derivatisation resulted in the lowest CV values at 150 W for 1.5 min and for

adapted microwave-assisted derivatisation, this was true at 350 W for 3.0 min. This study demonstrates that derivatisation of organic acids should be done with care as the derivatisation parameter intervals (or the exactness thereof) largely influences the repeatability.

KEY TERMS: Derivatisation; gas chromatography-mass spectrometry; methoxymation; microwave-assisted derivatisation; organic acids; silylation.

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ABBREVIATIONS

A:

Ace	N-acetyl-L-alanine
Aco	Cis-aconitic acid
Adi	Adipic acid
AM	Adapted microwave-asssisted derivatisation
AT	Adapted thermal derivatisation

B:

BSA	Bistrimethylsilylacetamide
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
BSTFA-TMCS (99:1%)	N,O-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane

C:

C	Carbon
CAS	Chemical abstracts service
$CHCl_3$	Chloroform
Cit	Citric acid
CV	Coefficient of variance
C19:0Me	Methyl nonadecanoate

E:

EI	Electron impact

F:

Fig.	Figure
Figs.	Figures
Fum	Fumaric acid

G:

GC-MS	Gas chromatography-mass spectrometry

H:

Hip	Hippuric acid

HMDS	Hexamethyldisilzane
H_2O	Water
HPLC-MS	High performance liquid chromatography-mass spectrometry

I:

i.e.	Id est; that is

K:

Ket	2-Ketoglutaric acid

L:

LLE	Liquid-liquid extraction

M:

1M	Methoxymated
M	Microwave-assisted derivatisation
Mal	Malonic acid
MeOH	Methanol
MeOX	Methoxyamination solution
MS-MS	Tandem mass spectrometry
MSTFA	N-methyl-(trimethylsilyl)trifluoroacetamide
MTBFA	N-methyl-bis(trifluoroacetamide)
MTBSTFA	N-methyl-N-t-butyldimethylsilyltri-fluoroacetamide
MATLAB	Matrix laboratory

N:

NaOH	Sodium hydroxide
NIST	National institute of standards and Technology
NWU	North-West University

O:

Oro	Orotic acid
Oxa	Oxalic acid

P:

Pal	Palmitic acid

PCA	Principal component analysis
Pen	Pentadecanoic acid
PFBCl	Pentafluorobenzoyl chloride
PFPOH	Pentafluoropropanol
Pyr	Pyruvic acid
PTFE	Polytetrafluoroethylene

S:

Seb	Sebacic acid
SPE	Solid phase extraction
SIM	Single ion monitoring
Suc	Succinylacetone

T:

TAA	Tetra-alkylammonium
TCA	Tricarboxylic acid
T	Thermal derivatisation
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl
TMS-DEA	Trimethylsilyldiethylamine
TMSI	Trimethyl-silylimidazole

Symbols

α	Alpha
ß	Beta
°C	Degrees Celsius
$-(CH2)_n$	Side chain
-CONH	Amide group
-COOH	Carboxyl group
-H	Hydrogen
-NH-	Amino group
=O	Keto group
-OH	Hydroxyl group
%	Percentage
-SH	Thiol group
$-Si(CH_3)_3$	Trimethylsilyl group

T^2	T-squared

Measuring units

h	Hour(s)
°C	Degrees Celsius
m	Meter
mm	Millimetre
min	Minute(s)
mg	Milligram
ml	Millilitre
ms	Milliseconds
m/z	Mass-to-charge ratio
psi	Pounds per square inch
µg	Microgram
µl	Microliter
µm	Micrometer
<	Less than
>	Greater than
≤	Less than or equal to
V	Volt
W	Watt

CHAPTER 1: INTRODUCTION

1.1 Background

Organic acids are weak acids that originate exogenously, from diet and medication, or endogenously from a variety of metabolic pathways including the tricarboxylic acid (TCA) cycle, fatty acid beta (β)-oxidation, neurotransmitter turnover, microorganisms and carbohydrate, protein and ketone body metabolism. The measurement of organic acids in biological fluids make it possible to obtain a representative endo- and exogenous metabolic profile that has developed into a powerful tool for assessing amongst other things, health status, nutritional status, vitamin deficiencies, response to xenobiotics and to screen for inborn errors of metabolism (Tsoukalas *et al.*, 2017, Gallagher *et al.*, 2018).

In a clinical environment, gas chromatography-mass spectrometry (GC-MS) is the gold standard method for the measurement of especially organic acids (Fiehn, 2017). The common use of gas chromatography can be linked to its advantages which include: high chromatographic resolution, easy linkage with sensitive and selective detectors, less expensive instrumentation, ability to separate a wide variety of compounds and the fact that it is relatively fast and simple to use (Sneddon *et al.*, 2007, Farajzadeh *et al.*, 2014). For GC-MS analysis of organic acids, derivatisation prior to analysis is usually necessary to substitute active hydrogens occurring in functional groups that may include hydroxyl (-OH), carboxyl (-COOH), amino (-NH-), thiol (-SH) and phosphate groups (Parkinson, 2014). Derivatisation have the ability to improve volatility, enhance separation, improve selectivity and to gain thermal stability (Orata, 2012). These improvements are important, since gas chromatography separates compounds according to their interaction with the capillary column that is influenced by the boiling points and polarity of compounds (Viant and Sommer, 2013).

1.2 Problem statement

In the broad organic acid class classification, different combinations of functional groups exist that each prefer different optimal derivatisation conditions. Several different reagents and parameters can be used for derivatisation procedures resulting a compromise between maximisation of the reaction efficiency and minimisation of the formation of unexpected side products (Gullberg *et al.*, 2004). Amongst other parameters derivatisation is very dependent on temperature and reaction time to result in an efficient derivatisation reaction (Christou *et al.*, 2014). Inadequate reaction time often results in incomplete derivatisation that leads to the formation of multiple peaks for the same compound that is not ideal for the identification of organic acids in low physiological concentrations (Little, 2014). Furthermore, unsuitable temperature have the consequences of decomposed

compounds or incomplete derivatisation (Parkinson, 2014). These undesirable conditions complicate accurate and repeatable measurement of organic acids (Little, 2014).

Having an inborn error of metabolism requires early, rapid and accurate diagnosis in order to have a chance to prevent disability and death. These requirements keep the use of GC-MS as a first-tier laboratory screening test in the spotlight (Hampe *et al.*, 2017). Considering the importance of concise reproducible GC-MS results in a clinical environment it is crucial to investigate and standardise the procedures to which the sample of interest are subjected to prior GC-MS analysis. Protocols for organic acid analysis by GC-MS can easily be obtained in literature, however established protocols or standard methods for reliable organic acid derivatisation is somewhat lacking (Table 2.1). Resultantly some important questions are now asked with this study, i.e. how does different temperatures, reaction times, methoxymation and microwave-assistance, influence the derivatisation outcome of organic acids?

1.3 Aim and objectives

The **aim** of this investigation was to systematically compare the outcome of predefined derivatisation parameters required for thermal- and microwave-assisted derivatisation as a prerequisite for GC-MS analysis of selected organic acids.

Specific **objectives** followed to achieve this aim:

- A literature-based investigation of derivatisation temperatures, microwave energies and reaction times used, resulting in the selection of parameters to be compared for the derivatisation of selected organic acids.
- Compare parameters within specific conditions for thermal-, adapted thermal-, microwave-assisted- and adapted microwave-assisted derivatisation for selected individual organic acid derivatives.
- Compare repeatability within and between conditions for thermal, adapted thermal, microwave-assisted and adapted microwave-assisted derivatisation types for selected individual organic acid derivatives.
- Comparing the most repeatable derivatisation condition for a selected group of organic acid derivatives.

1.4 Structure of dissertation

This compilation of five chapters is written in dissertation format according to the requirements for a Master study in Biochemistry at the North-West University. **Chapter one**, the current chapter, introduces the study through a brief overview of the background, problem statement which includes

the motivation for this study, aim, objectives and also the structure layout of this dissertation. **Chapter two** gives a detailed overview of the biological importance of organic acids, information about GC-MS and reviews the main chemical derivatisation procedures available. This chapter furthermore includes a summary of silylation conditions currently used for the measurement of organic acids and contains information regarding the organic acids chosen for this study. **Chapter three** contains all the general materials and methods used for this comparison study, followed by the experimental approach. **Chapter four** presents and discuss the results obtained in this study as comparisons within and between the four derivatisation types (thermal, adapted thermal, microwave-assisted and adapted microwave-assisted). **Chapter five** provides a general conclusion from all comparisons and future recommendations emanated from the results. This is followed by the **references** used in this dissertation. **Appendix A** gives supplementary information from Chapter 4 that includes, the principal component analysis and Hotelling's T-squared figures for additional conditions investigated, as a mean for outlier evaluation. **Appendix B** contains cumulative distribution plots of all included organic acids at all conditions within a derivatisaiton type to compare coefficient of variance results.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

The measurement of organic acids gained interest in 1963 when the first study of organic acid metabolism was reported by Klenka and Kahlke. The study aimed to identify phytanic acid in a patient with Refsum's disease with the use of mass spectrometry (Kaluzna-Czaplińska, 2011). Research continued and in 1966 the first of many organic acid metabolic disorders, isovaleric acidemia, was discovered with the use of gas chromatography-mass spectrometry (GC-MS) (Tanaka and Isselbacher, 1967, Zhang *et al.*, 2000). With GC-MS as the longest established instrumental chromatographic technique, great effort has already been made to develop different chemical derivatisation procedures in order to improve analysis of organic acids (Husek, 2000).

2.2 Organic acids

Organic acids consist of a broad class of highly water-soluble compounds that contain at least one carboxylic group (as shown in Fig. 2.1) and have relatively low molecular weights, less than 300 molar (Kumps *et al.*, 2002). Organic acids can also consist side chains, hydroxyl, hydrogen, or keto-functional groups (Rinaldo, 2008). The broad class of organic acids can further be classified according to their chemical structure, biological distribution or physiological significance (Bouatra *et al.*, 2013). Organic acids are weak acids that originate exogenously (diet and medication) or endogenously from a variety of sources including the tricarboxylic acid (TCA) cycle, fatty acid beta (β)-oxidation, turnover of neurotransmitters, microorganisms, and metabolism of carbohydrates, proteins and ketones in the body (Fig. 2.2) (Tsoukalas *et al.*, 2017, Gallagher *et al.*, 2018). The metabolic activity of these pathways are generally reflected through the presence and amount of organic acids in biological fluids (Gallagher *et al.*, 2018).

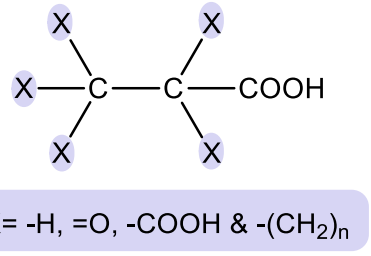


Figure 2.1: Structure of common organic acids. Organic acids can consist of the following functional groups: hydrogen (-H), keto (=O), hydroxyl (-OH), carboxyl (-COOH) or side chain (-$(CH_2)_n$) with at least one carboxylic group. Adapted from Rinaldo, 2008.

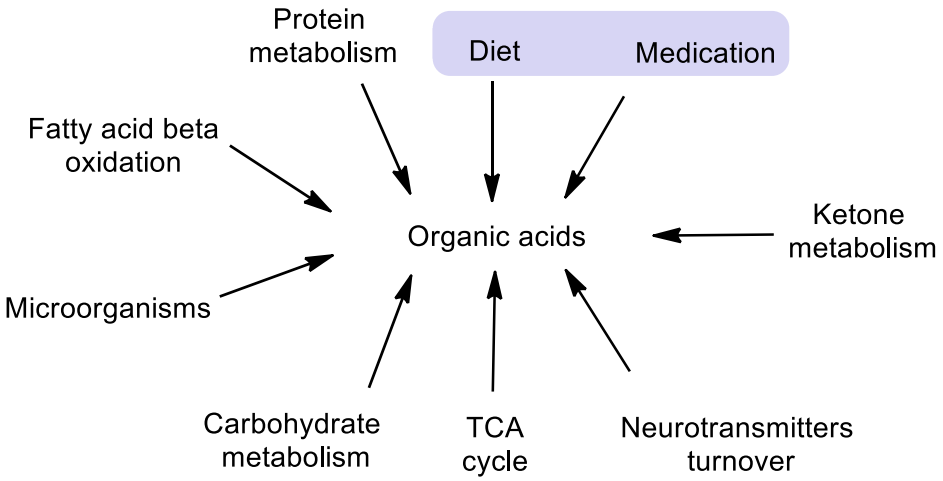


Figure 2.2: Endo- and exogenous origin of organic acid in biological fluids. Diet and medication are responsible for the exogenous origin and all the other are endogenous sources. Adapted from Gallagher *et al.* (2018) and Tsoukalas *et al.* (2017).

Organic acids can be detected in a variety of biological fluids including urine, blood, saliva, cerebrospinal- and amniotic fluid (Chace, 2001). From these, urine is the most common specimen since it is easy to collect non-invasively, is adequate for analysis, lacks protein and has the highest organic acid concentration (Gallagher *et al.*, 2018). Up to 500 organic acids can be identified in a urine sample (Whiteley *et al.*, 2009), from which more than 100 organic acids are excreted in abnormal amounts in cases where organic aciduria is present (Villani *et al.*, 2017). Qualitative or quantitative GC-MS urinary organic acid profiles make future evaluation possible, especially when assessing health status, nutritional status, vitamin deficiencies, response to xenobiotics and to screen for inborn errors of metabolism (Tsoukalas *et al.*, 2017).

In order to prepare urine samples for organic acid analysis, extraction of organic acids are needed to remove potential interferences (Vas *et al.*, 2008). Two frequently used extraction procedures are solid phase extraction (SPE) and liquid-liquid extraction (LLE) (Kumari *et al.*, 2011). The use of LLE is a relatively simple method to extract organic acids from urine and uses an acidification step to decrease organic acid solubility in the aqueous phase, allowing organic acid separation to the more apolar organic phase (ethyl acetate and diethyl ether) (Villani *et al.*, 2017). Following the extraction procedure, organic acids are derivatised before GC-MS analysis (Christou *et al.*, 2014).

2.3 Gas chromatography-mass spectrometry

The coupling of gas chromatography with mass spectrometry was first achieved in 1957 (Harvey, 2017). Until today the gas chromatograph is still utilised to separate a variety of compounds according to their interaction with the stationary phase of the selected capillary column. The boiling point and polarity of a compound will influence the retention on the column (Kaluzna-Czaplińska, 2011). Chromatographic separation leads to compounds that elute at different times from the

column, allowing the mass spectrometer to detect the molecules separately (Harvey, 2017). Other analytical techniques available include high performance liquid chromatography (HPLC-MS) and tandem-mass spectrometry (M-MS) (Tsoukalas *et al*., 2017). However, the use of gas chromatography include the following advantages: high chromatographic resolution, easy linkage with sensitive and selective detectors, less expensive instrumentation, ability to separate a wide variety of compounds and the fact that it is fast and simple to use (Sneddon *et al.*, 2007, Farajzadeh *et al.*, 2014).

2.4 Derivatisation

Since gas chromatography analysis started to dominate chromatographic separation in the 1950s, great measures were taken to develop procedures that will enhance volatility and thermal stability of polar compounds to be more suitable for gas chromatography analysis. Research was almost entirely aimed at substituting active hydrogen atom(s), through the use of reagents, to obtain derivatives with less active functional groups, giving rise to different chemical derivatisation procedures (Husek, 2000).

Organic acids are very polar and thermally unstable metabolites making them unsuitable for gas chromatography separation without derivatisation (Kaluzna-Czaplińska, 2011). Derivatisation reagents transform the chemical structure of organic acids to obtain increased volatility, enhanced separation, improved selectivity and thermal stability. Derivatisation for GC-MS can be performed by various procedures, but the main chemical reactions include acylation, alkylation and silylation (Orata, 2012).

2.4.1 Gas chromatography-mass spectrometry derivatisation

2.4.1.1 Acylation

Acylation is a reaction in which an acyl group is introduced to an organic compound and the loss of a hydroxyl group occurs. Figure 2.3 illustrates the acylation reaction where a hydroxyl group is derivatised by using acetic anhydride. Other available acylation reagents include: fluorinated anhydrides, fluoroacylimidazoles, pentafluorobenzoyl chloride (PFBCI), pentafluoropropanol (PFPOH) and N-methyl-bis(trifluoroacetamide) (MBTFA). Acylation is used when improved chromatographic separation of especially sugars and reduced wide-ranging adsorption effects are necessary (Orata, 2012). Although acylation holds significant advantages, the reagents are hazardous, sensitive to moisture and can cause the formation of by-products (Lai and Fiehn, 2016).

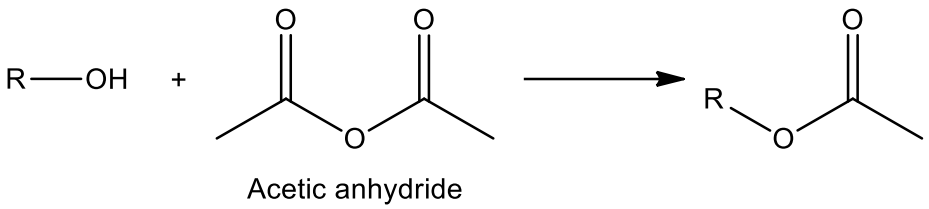


Figure 2.3: A common acylation reaction. The derivatisation of a hydroxyl group by using acetic anhydride (Lai and Fiehn, 2016).

2.4.1.2 Alkylation

Alkylation, an esterification reaction, entails the substitution of an active hydrogen by an aliphatic or aliphatic-aromatic (alkyl) group (Orata, 2012). The active hydrogen occurs in the hydroxyl (-OH), thiol (-SH), amino (-NH-), amide (-CONH-) and carboxyl (-COOH) polar groups (Wang *et al.*, 2013). This chemical reaction provides stable organic acids which is an advantage if storage for extended periods are necessary (Parkinson, 2014). Reagents used for alkylation may involve diazomethane, alkyl halides, alcohols and tetra-alkylammonium (TAA) salts (Wang *et al.*, 2013). A general alkylation example with diazomethane as reagent is shown in Figure 2.4. Nowadays the use of alkylation is avoided, due to its unstable and highly carcinogenic reagents (Husek, 2000).

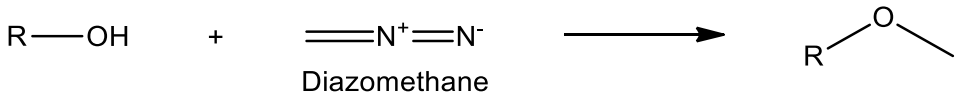


Figure 2.4: General reaction for alkylation. The alkylation of a hydroxyl group with diazomethane (Lai and Fiehn, 2016).

2.4.1.3 Silylation

Silylation has been the most commonly used derivatisation method for urinary organic acids since silylation reagents were introduced in the 1960s (Husek, 2000). Silylation entails the addition of a trimethylsilyl (TMS) group that usually substitutes active hydrogens. The reactivity of a silyl group towards a functional group is as follows: alcohol> phenol> acid> amine> amide/ hydroxyl. Silylation reagents have the ability to derivatise a broad spectrum of compounds, volatilise compounds without difficulty and yield narrow, symmetrical peaks (Orata, 2012). Silylation reagents include amongst others: Bistrimethylsilylacetamide (BSA), N-methyl-trimethylsilyltrifluoroacetamide (MSTFA), N-methyl-N-t-butyldimethylsilyltri-fluoroacetamide (MTBSTFA), hexamethyldisilzane (HMDS), trimethyl-silylimidazole (TMSI), trimethylsilyldiethylamine (TMS-DEA), halo-methylsilyl reagents, trimethylchlorosilane (TMCS) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Kaluzna-Czaplińska, 2011). The choice of silylation reagent depends on the selected compound's characteristics such as reactivity, selectivity, volatility and stability (Parkinson, 2014).

A study by Moros *et al.* (2017) identified MSTFA compared to BSTFA as the reagent to supply the most repeatable derivatisation, most identified metabolites and larger peak areas. The study

concluded that MSTFA with 1% TMCS catalyst provided even better results. The addition of TMCS is responsible for catalysing derivatisation of hindered functional groups and to increase TMS donor potential (Kouremenos *et al.*, 2010a, Parkinson, 2014). The combination of N,O-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane [BSTFA-TMCS (99:1%)] is also widely recommended and frequently used (Wajner *et al.*, 2009, Nakagawa *et al.*, 2010, Xiong *et al.*, 2015). BSTFA is a sufficiently volatile reagent that interferes minimally with early eluting compounds. Derivatives formed by BSTFA also have the benefit of not containing any active hydrogens that otherwise lead to artefact formation (Kouremenos *et al.*, 2010a). Silylation with BSTFA-TMCS (99:1%) of organic acid functional groups are illustrated in Figure 2.5.

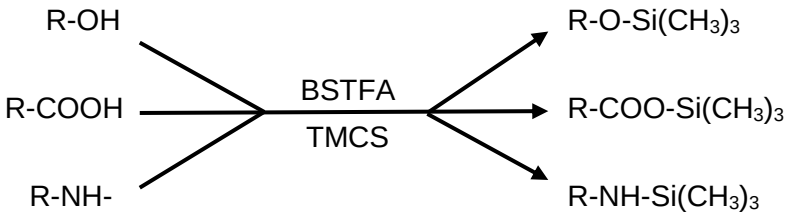


Figure 2.5: Silylation of possible organic acid functional groups. Derivatisation of hydroxyl, carboxyl and amino functional groups using BSTFA-TMCS (99:1%). Adapted from Kouremenos *et al.* (2010a).

2.4.2 Derivatisation drawbacks

Although derivatisation is necessary and has many advantages for GC-MS analysis of organic acids, the process is time consuming, labour intensive and in some circumstances results in the formation of multiple derivatives for the same compound. The latter will result in multiple peaks on a chromatogram that will complicate compound detection (Little, 2014). The formation of multiple derivatives can be caused by insufficient or undesirable derivatisation conditions, steric hindrances or keto- groups that can (or cannot) be silylated in the same sample (Gerlo *et al.*, 2006, Little, 2014). Insufficient derivatisation may cause multiple derivatives for a compound, due to different trimethylsilyl binding sites that silylate non-systematically and only partially. Artefacts can also form due to a derivatisation reagent that binds to itself, other organic or inorganic reagents or even solvents that are present in a sample or glassware (Little, 2014). To prevent artefact formation, satisfactory derivatisation is a necessity. The main problem is that exact silylation conditions (reaction time and temperature) required for a variety of organic acids are still unknown (Parkinson, 2014, Moros *et al.*, 2017). A literature overview of current silylation conditions performed with the use of BSTFA-TMCS (99:1%) are summarised in Table 2.1. This table shows that a variety of silylation temperatures (from 50 to 90°C) and reaction times (from 30 to 120 min) can be used when derivatising samples.

Table 2.1: Literature based thermal silylation conditions

Compounds of interest	Silylation [BSTFA-TMCS (99:1%)]			References
	Solvent	Temperature (°C)	Reaction time (min)	
Urinary organic acids	-	80	30	(Rinaldo, 2008)
Urinary organic acids	-	70-80	30-120	(Jones and Bennett, 2010)
Plasma metabolites	Pyridine	70	60	(Hong *et al.*, 2012)
Urinary organic acids	Pyridine	85	45	(Reinecke *et al.*, 2012)
Organic acid standards	-	50	30	(Christou *et al.*, 2014)
Urinary organic acids	Pyridine	70	30	(Yi *et al.*, 2014)
Urinary organic acids	Pyridine	80	45	(Vasquez *et al.*, 2015)
Urinary organic acids	Pyridine	60	60	(Mason *et al.*, 2016)
Urinary organic acids	Pyridine	70	45	(Irwin *et al.*, 2018)
Urinary organic acids	-	65-90	10-30	(Gallagher *et al.*, 2018)

It is important to note that the silyl reagents are moisture sensitive and may have an adverse effect on the reaction (Orata, 2012). A potential drawback of derivatisation reactions using silyl reagents in combination with pyridine is that pyridine is hygroscopic and special care should be taken to keep it anhydrous. Pyridine is used as a solvent and acts as an acid acceptor for the protons displaced during derivatisation, thus driving the reaction. Long incubation periods are used in an attempt to derivatise hindered functional groups and to ensure complete derivatisation of compounds (Casals *et al.*, 2014). This is not always a practical option in a routine clinical laboratory due to time constraints and urgency of results (Hampe *et al.*, 2017).

2.4.3 Improving derivatisation

Even though derivatisation parameters can never be optimal for all the compounds of interest, it is still necessary to consider different derivatisation reagents, solvents, temperatures and reaction times in the decision-making process towards the improvement of derivatisation (Parkinson, 2014, Moros *et al.*, 2017). These parameters are affected by chemical and physical characteristics of compounds that influence, amongst others, the reaction rate and also the stability of derivatives (Parkinson, 2014). The use of ethoxymation, oxymation or methoxymation in addition to typically used derivatisation reagents, is highly recommended for aldehyde or keto- acids (Kanani *et al.*, 2008). Another possibility to consider is the use of microwave-assisted derivatisation for a faster and more effective process (Chung *et al.*, 2008).

2.4.3.1 Ethoxymation, oxymation and methoxymation

Keto- groups can form multiple derivatives due to keto- enol tautomerisation that may occur that enable an additional TMS group to attach (Qiu and Reed, 2014). The example in Figure 2.6 shows the formation of two derivatives instead of one for pyruvic acid. The need for additional sample preparation can be considered if a sample contains compounds with keto- or aldehyde groups (Kanani *et al.*, 2008). Ethoxymation, oxymation or methoxymation can be used to stabilise keto- and aldehyde groups for the purpose of reducing the number of derivatives which form during silylation

(Kumari *et al.*, 2011). Figure 2.7 illustrates the general reaction of methoxymation stabilisation of a keto- group. For organic acid analysis, an adapted sample preparation method including oxymation for keto- acids, will especially be valuable since multiple keto- acids are used as markers for energy and amino acid metabolism (Nguyen *et al.*, 2013). Reagents such as: ethylhydroxylamine hydrochloride (ethoxymation), hydroxylamine hydrochloride (oxymation) and methylhydroxylamine hydrochloride (methoxymation) can be used to prevent formation of multiple peaks on a chromatogram for a single compound (Kaluzna-Czaplińska, 2011). According to Fiehn *et al.* (2000) and Ruiz-Matute *et al.* (2011) methoxymation is the more suitable option for identification of compounds, compared to oxymation. The most common methoxymation conditions used before silylation with BSTFA-TMCS (99:1%) is 30°C for 90 min with methylhydroxylamine hydrochloride dissolved in pyridine (20 mg/ml) (Abbiss *et al.*, 2015, Fiehn, 2017).

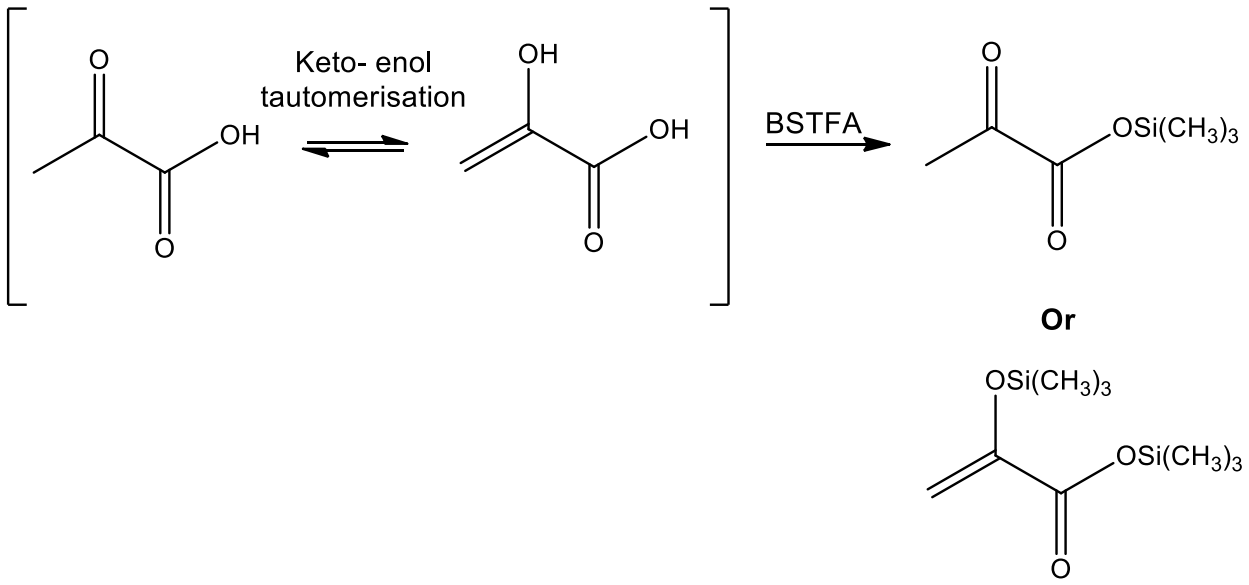


Figure 2.6: **Silylation of pyruvic acid consisting of a keto- group.** Formation of two TMS derivatives for pyruvic acid due to keto- enol tautomerisation. Adapted from Little (2014).

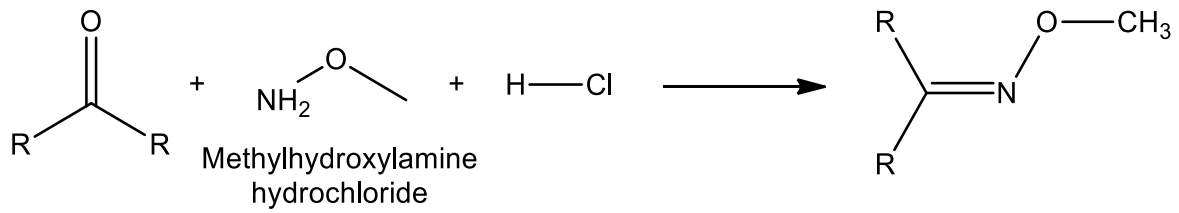


Figure 2.7: Reaction scheme of methoxymation. A keto- group treated with methylhydroxylamine hydrochloride preventing binding of a TMS group (Lai and Fiehn, 2016).

2.4.3.2 Microwave-assisted derivatisation

Microwave-assisted derivatisation ensures faster and more effective reactions. Microwave is a form of electromagnetic energy that penetrates into reaction vessels, directly transferring energy to

molecules, thereby reducing energy transfer time and making the procedure more efficient (Chung *et al.*, 2008). A study by Khoomrung *et al.* (2015) found no significant difference in the metabolite profiles between thermal and microwave-assisted derivatisation. Another study (Chung *et al.*, 2008) claimed the opposite, indicating that microwave energy and time influenced derivatisation efficiency to such an extent that metabolite profiles were affected. Microwave energy and reaction time are two parameters with microwave-assistance that can be investigated to improve derivatisation (Kouremenos *et al.*, 2010a). In Table 2.2 some published microwave-assisted derivatisation conditions are summarised. A variety of microwave energies from 150 to 450 W and reaction times, from 1.5 to 3.0 min were used. A review of published applications with the use of microwave-assisted derivatisation for GC-MS analysis emphasises that despite the potential advantages, lack of investigation and limited use is observed (Soderholm *et al.*, 2010).

Table 2.2: Literature based microwave-assisted silylation conditions

Compounds of interest	Silylation [BSTFA-TMCS (99:1%)]			References
	Solvent	Microwave energy (W)	Reaction time (min)	
Organic acid standards	Pyridine	150	1.5	(Kouremenos *et al.*, 2010a)
Urinary organic acids	-	450	1.5	(Kouremenos *et al.*, 2010b)
Methylmalonic acid in serum	-	400	2.0	(Ye *et al.*, 2010)
Plasma metabolites	Pyridine	230	3.0	(Hong *et al.*, 2012)

2.5 Evaluating derivatisation parameters

A study by Bowden *et al.* (2009) investigated optimal GC-MS derivatisation conditions of steriods including temperature, microwave-assistance and reaction time. These parameters were compared by means of reproducibility between replicates, normalised relative response values, calculated by dividing the peak area of each compound with the peak area of an internal standard and considered derivatisation time. In another study, optimum derivatisation temperature and time were investigated through a similar performance criterion evaluating also the relative response to determine reaction yield and repeatability by determination of coefficient of variance (CV) (Christou *et al.*, 2014). These performance criteria were used as a guideline for this study to determine the effect of changes in derivatisation parameters. The performance criteria for this investigation included repeatability, relative response and derivatisation reaction time (as explained in the result section, Chapter 4). Of these criteria, repeatability was the focus.

2.6 Selecting compounds for derivatisation parameter comparison

Considering that derivatisation efficiency of organic acids is influenced by the combination of functional groups, it is necessary to evaluate each organic acid separately, but also as a mixture of related and unrelated functional groups (Little, 2014). It is important to ensure different functional group combinations are included when determining preferred derivatisation conditions for organic

acids (Koek *et al.*, 2011, Christou *et al.*, 2014). As such, the compounds specifically selected for derivatisation comparisons in this study will be described here.

Organic acids consist of at least one carboxyl group, side chains, hydroxyl, hydrogen or keto-, functional groups (Rinaldo, 2008). For the purpose of this study, organic acids were selected by means of functional groups, chain length, presence of aromatic ring structures and physiological concentration given in Table 2.3 and Table 2.4, as originally obtained from the Human Metabolome Database (Wishart *et al.*, 2018). Eight monocarboxylic acids (hippuric acid, N-acetyl-L-alanine, orotic acid, palmitic acid, pentadecanoic acid, 3-phenylbutyric acid, pyruvic acid and succinylacetone), six dicarboxylic acids (adipic acid, fumaric acid, 2-ketoglutaric acid, malonic acid, oxalic acid and sebacic acid) and two tricarboxylic acids (cis-aconitic acid and citric acid) in combination with other functional groups and/ or aromatic ring structures acids (hippuric acid, orotic acid and 3-phenylbutyric acid) were selected to represent the broader organic acid class. From these selected compounds, hippuric acid, 2-ketoglutaric acid, orotic acid, pyruvic acid and succinylacetone represented keto-acids, enabling us to compare the effect of methoxymation.

Table 2.3: Chemical structures of organic acids selected for derivatisation evaluations

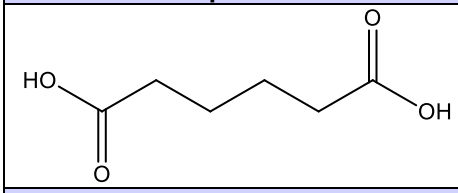
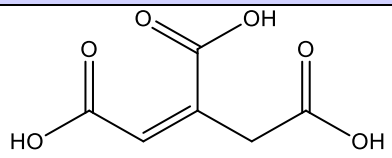
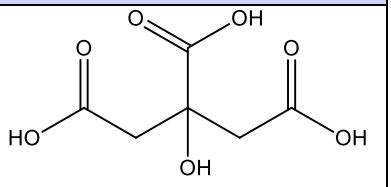
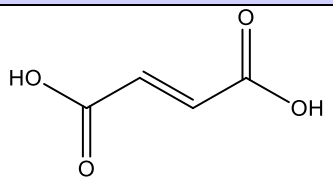
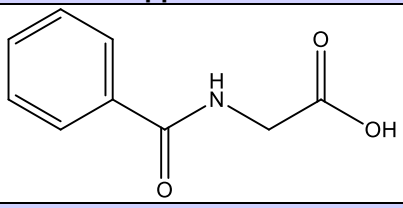
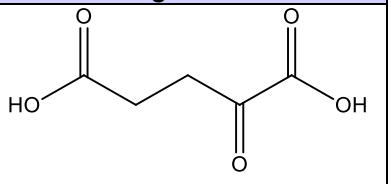
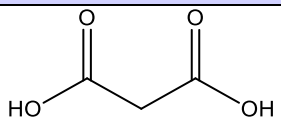
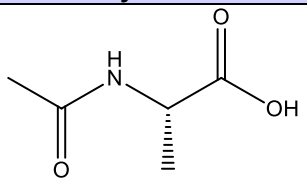
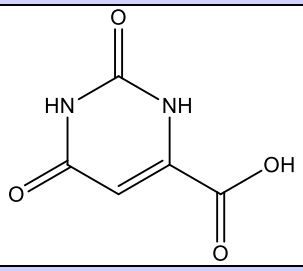
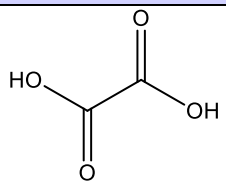
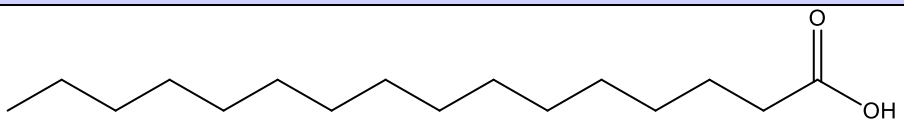
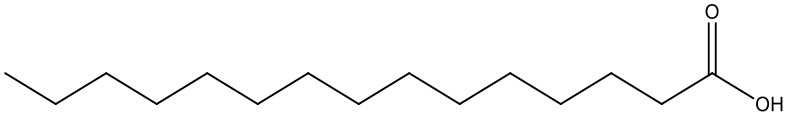
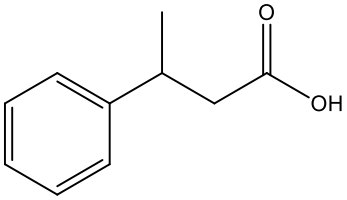
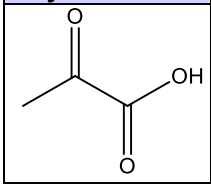
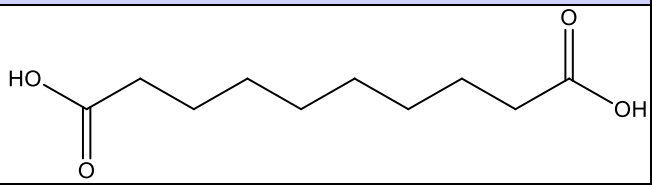
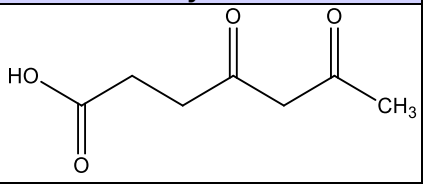
Adipic acid	**Cis-aconitic acid**	**Citric acid**
		
Fumaric acid	**Hippuric acid**	**2-Ketoglutaric acid**
		
Malonic acid	**N-acetyl-L-alanine**	**Orotic acid**
		
Oxalic acid	**Palmitic acid**	
		
Pentadecanoic acid	**3-Phenylbutyric acid**	
		
Pyruvic acid	**Sebacic acid**	**Succinylacetone**
		

Table 2.4: Properties of compounds selected for derivatisation evaluations

Organic acid	CAS number	Molecular weight (g/mol)	Organic acid sub class	Functional groups				Physiological concentration (µg/ml)	Solvent
				R-COOH	R-OH	R=O	R-NH-R		
Adipic acid	124-04-9	146.142	Dicarboxylic acid	2				7.45 (1.17 - 51.15)	Water
Cis-aconitic acid	585-84-2	174.108	Tricarboxylic acid	3				22.64 (4.70 - 76.61)	Water
Citric acid	77-92-9	192.123	Tricarboxylic acid	3	1			+/- 300.00	Water
Fumaric acid	110-17-8	116.072	Dicarboxylic acid	2				0.93 - 2.09	Water
Hippuric acid	495-69-2	179.175	Monocarboxylic acid	1		1	1	388.84 (50.17 - 1093.06)	Water
2-Ketoglutaric acid	328-50-7	146.098	Dicarboxylic acid	2		1		< 109.58	Water
Malonic acid	141-82-2	104.062	Dicarboxylic acid	2				+/- 0.62	Water
N-acetyl-L-alanine	97-69-8	131.131	Monocarboxylic acid	1		1	1	-	Water
Orotic acid	65-86-1	156.097	Monocarboxylic acid	1		2	2	0.25 +/- 0.19	Water
Oxalic acid	144-62-7	90.034	Dicarboxylic acid	2				7.38 (3.51 - 12.61)	Water
Palmitic acid	1957/10/03	256.430	Monocarboxylic acid	1				28.21 (15.39 - 58.98)	Methanol
3-Phenylbutyric acid	4593-90-2	164.204	Monocarboxylic acid	1				-	Methanol
Pyruvic acid	127-17-3	88.062	Monocarboxylic acid	1		1		1.85 (0.88 - 3.26)	Water
Sebacic acid	111-20-6	202.250	Dicarboxylic acid	2				4.05 - 10.11	Methanol
Succinylacetone	51568-18-4	158.153	Monocarboxylic acid	1		2		4.43 (0.95-7.43)	Water
Internal standard									
Nonadecanoic acid, Methyl ester	173-94-8	312.538	Not organic acid					-	2,2,4-trimethylpentane
Pentadecanoic acid	1002-84-2	247.403	Monocarboxylic acid	1				-	Chloroform

A standard solution instead of a biological matrix sample was selected for this comparison study in order to monitor the derivatisation efficiency of certain organic acids without other influences such as extraction efficiency (Bouatra *et al.*, 2013, Moros *et al.*, 2017). For investigations using biological samples Wishart *et al.* (2018) suggested concentration ranges of about 0.24 µg/ml for orotic acid and 389 µg/ml for hippuric acid and Nakagawa *et al.* (2010) reported a 50 µg/ml organic acid addition to samples as representative of normal biological concentration. For this study, the final concentration for each of the organic acids evaluated was 20 µg/ml.

It is also important to include an internal standard when investigating method parameters as this can correct losses during sample preparation for specific compounds and be used for quality control purposes (Fiehn, 2017). A commonly used internal standard for GC-MS analysis of primarily organic acids is 3-phenylbutyric acid (Reinecke *et al.*, 2012, Tran *et al.*, 2014). Considering that this is a well-used internal standard, this organic acid was included as a compound of interest and not as an internal standard. For an internal standard influenced by derivatisation we included pentadecanoic acid as recommended by literature (Kaluzna-Czaplińska, 2011, Christou *et al.*, 2014, Moros *et al.*, 2017). Nonadecanoic acid, methyl ester (C19:0Me) was included in this study as an internal standard not influenced by derivatisation or methoxymation (Fiehn, 2017).

CHAPTER 3: MATERIALS AND METHODS

3.1 Introduction

Derivatisation forms part of the sample preparation procedure in order to transform the chemical structure of organic acids. For gas chromatography-mass spectrometry (GC-MS) analysis of organic acids, derivatisation is required prior to analysis in order to improve the volatility, separation, selectivity, solubility and thermal stability (Orata, 2012). Although silylation is a commonly used method to derivatise organic acids, there is a lack of established or standardised silylation methods in literature. Problems experienced with silylation such as formation of multiple derivatives, can possibly be prevented with optimal derivatisation conditions. This chapter describes the detail regarding the: chemicals, solutions, and general materials; GC-MS parameters; and data processing used to investigate silylation of selected organic acids. Lastly, an overview of the experimental approach that was followed to compare different derivatisation conditions within this study will be described in Section 3.6.

3.2 Chemicals, solutions and general materials

3.2.1 Chemicals

The following analytical standards were obtained from Merck (Johannesburg, South Africa): adipic acid (A26357), cis-aconitic acid (A3412), citric acid (C2404), fumaric acid (F8509), hippuric acid (112003), 2-ketoglutaric acid (75890), malonic acid (M1296), N-acetyl-L-alanine (A4625), orotic acid (O2750), oxalic acid (41706), palmitic acid (P0500), pentadecanoic acid (W433400), 3-phenylbutyric acid (116807), pyruvic acid (P2256), sebacic acid (283258), succinylacetone (D1415) and methyl nonadecanoic acid (C19:0Me). Reagents and solvents also from Merck included: methylhydroxylamine hydrochloride (MeOX) (89803), N,O-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane [BSTFA-TMCS (99:1%)] (33155), sodium hydroxide (NaOH) (S8045), pyridine (270970), chloroform ($CHCl_3$) (1031228), hexane (34859) and 2,2,4-trimethylpentane (360066). Honeywell (Burdick & Jackson) solvents including: methanol (MeOH) (230-4), water (H_2O) (365-4) and isopropyl alcohol (323-4) were purchased from Anatech Instruments (Pty) Ltd (Johannesburg, South Africa).

3.2.2 General materials

Glassware was prepared by soaking the items overnight (16 h) in 15 g/1.5 L Alconox detergent, (Merck, Johannesburg, South Africa). After soaking, the glassware was rinsed thoroughly with warm

water, followed by 18.2 Mohm milliQ water and sequentially with methanol, hexane, isopropyl alcohol and again methanol.

Agilent 2 ml screw cap glass vials (5190-9062); 400 µl flat bottom inserts (5181-3377) and 9 mm blue screw caps with polytetrafluoroethylene (PTFE) septa (5185-5820) were purchased from Chemetrix (Johannesburg, South Africa). Glass Pasteur pipettes (Z628018) and 1.5 ml Eppendorf tubes (T9661) were purchased from Merck (Johannesburg, South Africa).

3.2.3 Preparation of solutions

3.2.3.1 Preparation of the compound stock solution

A stock mixture containing the selected organic acids adipic acid, cis-aconitic acid, citric acid, fumaric acid, hippuric acid, 2-ketoglutaric acid, malonic acid, N-acetyl-L-alanine, orotic acid, oxalic acid, palmitic acid, pentadecanoic acid, 3-phenylbutyric acid, pyruvic acid, sebacic acid and succinylacetone) was prepared in a similar procedure to stock solution used by Fiehn *et al.* (2017). Firstly, a solution (solution A) was prepared by mixing 100 ml water, 250 ml methanol and 100 ml isopropanol in a volumetric cylinder. Dissolved oxygen was removed by purging the solution for 5 min with nitrogen gas. Each of the 4 mg of each of the organic acids except for pyruvic acid sodium salt (5 mg), were separately weighed into individual tubes. All the organic acids were dissolved individually in 1.5 ml tubes, in either 1 ml methanol, water or chloroform according by their solubility (Table 2.4). Approximately 40 ml of solution A was transferred to a 200 ml volumetric flask whereafter all the dissolved organic acids were transferred from the tubes to the flask. The remaining solution A was used to repeatedly rinse the tubes and add them to the volumetric flask. The flask was filled to 200 ml with solution A, resulting in a final concentration of 20 µg/ml for each of the organic acids. The stock solution was stored in a freezer at -20°C until aliquoted into glass gas chromatography vials.

All aliquots were prepared in advance. The stock mixture was removed from the freezer and allowed to reach room temperature before being sonicated for 30 min. From the stock, 150 µl was aliquoted into 2 ml gas chromatography glass vials fitted with a 300 µl flat bottom inserts. The aliquots were then dried under a gentle stream of nitrogen, capped and stored at -20°C, until needed for experiments.

3.2.3.2 Preparation of the methyl nonadecanoate internal standard

The internal standard, methyl nonadecanoate (C19:0Me) was prepared by dissolving 1.5 mg of in 25 ml 2,2,4-trimethylpentane to obtain a final concentration of 0.4 mg/ml in a volumetric flask. The internal standard was vortexed and aliquoted into 2 ml glass vials, before it was stored at -20°C.

3.2.3.3 Preparation of methoxyamine solution

Methoxymation reagent was freshly prepared for each batch before analysis, by dissolving 20 mg methylhydroxylamine hydrochloride in 1 ml pyridine to obtain a concentration of 20 mg/ml. The solution was vortexed for one minute and then heated in an oven at 60°C for 15 min to ensure that the reagent was completely dissolved (Venter *et al.*, 2015).

3.3 Gas chromatography-mass spectrometry method standardisation

GC-MS instrument conditions were adapted from an in-house GC-MS method used for organic acid analysis at the North-West University (NWU) Metabolomics Platform. A single ion monitoring (SIM) method for the organic acid derivatives and C19:0Me were used to ensure a sensitive and rapid run time (Harvey, 2017). For quantification a characteristic ion with the highest response was selected for each derivative and for C19:0Me as a targeted ion (Table 3.1). The SIM method was created from full scan (50-550 m/z) data of individual standards and mixture samples. The National Institute of Standards and Technology (NIST) 2008 mass spectral library (Max Planck Institute, Golm, Germany) was used to compare mass spectra to distinguish between different derivatives for a specific compound. The SIM method included nine separate time segments corresponding to the retention times of the 32 organic acid derivatives and C19:0Me. A dwell time of 100 ms was used for each compound.

Table 3.1: Retention times and targeted ions used in single ion monitoring mode

Organic acid derivative	Derivative abbreviation	Retention time (min)	Targeted ion (m/z)
Pyruvic acid 1M 1TMS	Pyr 1M 1TMS	5.807	89
Pyruvic acid 2TMS	Pyr 2TMS	6.605	217
Oxalic acid 2TMS	Oxa 2TMS	7.050	219
Malonic acid 2TMS	Mal 2TMS	8.164	133
N-acetyl-L-alanine 1TMS	Ace 1TMS	8.728	188
N-acetyl-L-alanine 2TMS	Ace 2TMS	9.222	158
Fumaric acid 2TMS	Fum 2TMS	10.191	147
Malonic acid 3TMS	Mal 3TMS	11.249	133
3-Phenylbutyric acid 1TMS	Phe 1TMS	11.267	236
2-Ketoglutaric acid 2TMS	Ket 2TMS	11.354	157
2-Ketoglutaric acid 1TMS	Ket 1TMS	11.869	157
Adipic acid 2TMS	Adi 2TMS	12.124	111
2-Ketoglutaric acid 1M 2TMS a	Ket 1M 2TMS a	12.661	304
Succinylacetone 1M 1TMS a	Suc 1M 1TMS a	12.798	109
2-Ketoglutaric acid 1M 2TMS b	Ket 1M 2TMS b	12.932	304
Succinylacetone 1M 1TMS b	Suc 1M 1TMS b	12.972	109
Succinylacetone 1M 1TMS c	Suc 1M 1TMS c	13.046	109
Succinylacetone 1M 1TMS d	Suc 1M 1TMS d	13.121	109
Succinylacetone 2TMS a	Suc 2TMS a	13.287	169
2-Ketoglutaric acid 3TMS	Ket 3TMS	13.456	157
Succinylacetone 2TMS b	Suc 2TMS b	13.611	169
Succinylacetone 2TMS c	Suc 2TMS c	13.916	157
Succinylacetone 3TMS	Suc 3TMS	14.621	257
Orotic acid 3TMS	Oro 3TMS	15.267	357
Cis-aconitic acid 3TMS	Aco 3TMS	15.311	375
Citric acid 3TMS	Cit 3TMS	15.763	201
Hippuric acid 2TMS	Hip 2TMS	15.960	105
Hippuric acid 1TMS	Hip 1TMS	16.100	105
Citric acid 4TMS	Cit 4TMS	16.722	363
Sebacic acid 2TMS	Seb 2TMS	17.337	331
Palmitic acid 1TMS	Pal 1TMS	19.199	313
Internal standard			
Nonadecanoic acid, Methyl ester	C19:0Me	20.867	87
Pentadecanoic acid 1TMS	Pen 1TMS	18.059	299

3.3.1 GC-MS parameters

For the analysis an Agilent GC-MS instrument (Agilent Technologies, Wilmington, Delaware, United States of America) consisting of a 7890A GC system coupled to a 5975B inert XL mass selective detector was used. The gas chromatograph was equipped with a 7683B autosampler, split/ splitless injector and an Agilent DB-1MS UI column (30 m x 0.25 mm internal diameter x 0.25 µm film thickness) (Chemetrix, Johannesburg, South Africa). A sample volume of 1 µl was injected with a split ratio of 1:10. A constant pressure of 11.598 psi and inlet temperature of 250°C was used. Helium was the carrier gas with a flow rate of 1.315 ml/min. The oven temperature program started with an initial temperature of 60°C for 1 min, whereafter the temperature was increased to 185°C at a 10°C/ min rate where it remained for 2 min before it was further increased to 270°C at a rate of 11°C/ min providing a total analysis time of 23 min. A post-run of 4 min at 310°C was also included. The transfer line temperature was set to 290°C, the mass spectrometer ion source was maintained at 230°C and

the mass spectrometer quadrupole was set at 150°C. The mass spectrometer was used to operate in SIM mode (Table 3.1) with electron impact (EI) ionisation at 70 V.

3.4 Derivatisation procedure

A total of four derivatisation procedures were compared in this study, grouped into two main experimental groups: 1) Thermal derivatisation and 2) Microwave-assisted-derivatisation both containing a common and adapted derivatisation method (Fig. 3.1).

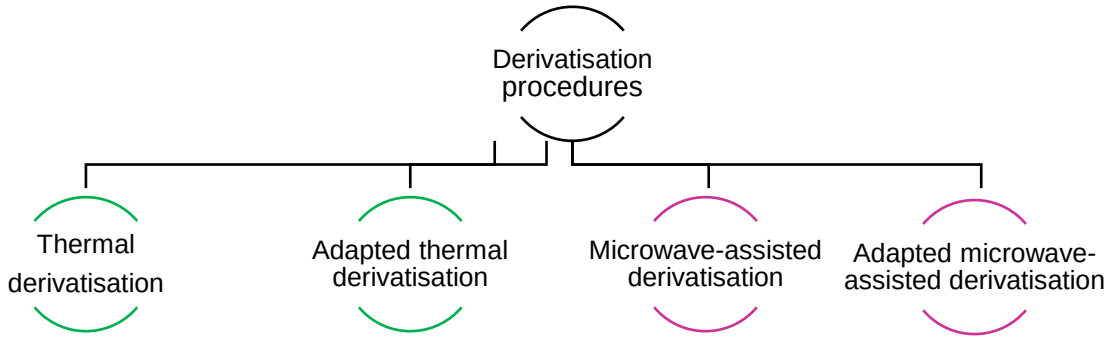


Figure 3.1: Derivatisation procedures used to derivatise organic acids. Comparing thermal derivatisation with adapted thermal derivatisation, followed by a comparison of microwave-assisted derivatisation with adapted microwave-assisted derivatisation.

3.4.1 Thermal derivatisation

For the thermal derivatisation part of this study, the dried organic acid standard mixture previously prepared (Section 3.2.3.1), was removed from the freezer and allowed to reach room temperature (25°C), followed by additional drying under a gentle stream of nitrogen at 37°C for 5 min. A volume of 50 µl pyridine, 50 µl BSTFA-TMCS (99:1%) and 50 µl C19:0Me (Section 3.2.3.2) were added to each vial, whereafter the vials were capped, vortexed and heated in an oven according to the temperature and reaction time combination being tested (Table 3.2). After the vials were heated in an oven the vials were allowed to cool for 10 min at room temperature, whereafter the samples were loaded for GC-MS analysis (Section 3.3.1).

Four different derivatisation temperature conditions were performed in the randomised order including 60, 85, 50 and 70°C respectively. For each of the selected temperatures, four different reaction times in the order of 30, 60, 90 and 120 min was performed as shown in Table 3.2.

All batches consisted of only one condition (specific temperature and specific reaction time combination). Randomisation within conditions were not possible due to restrictive instrumentation availability and time limitation for this investigation. In order to avoid possible effect of prolonged waiting time on the instrument, a time schedule for the derivatisation was used to eliminate batches waiting to be injected on the GC-MS.

Table 3.2: Batch sequence design for thermal derivatisation

	Thermal derivatisation															
Temperature (°C)	60				85				50				70			
Reaction time (min)	30	60	90	120	30	60	90	120	30	60	90	120	30	60	90	120
Batch sequence	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Replicates	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

3.4.2 Adapted thermal derivatisation

The adapted thermal derivatisation was performed with the organic acid mixture, prepared in advance (Section 3.2.3.1). The dried aliquots were removed from the freezer and allowed to reach room temperature, followed by drying under a gentle stream of nitrogen at 37°C for 5 min. Methoxymation, was performed with the addition of 50 µl methoxyamine solution (Section 3.2.3.3) to the vials, capped and heated in an oven at 30°C for 90 min. The vials were allowed to cool down for 10 min at room temperature whereafter the silylation procedure followed. Silylation involved the addition of 50 µl BSTFA-TMCS (99:1%) and 50 µl C19:0Me internal standard (Section 3.2.3.2). The vials were capped, vortexed and heated in an oven according to the specific batch conditions (Table 3.3). After incubation the vials were allowed to cool for 10 min and loaded for GC-MS analysis (Section 3.3.1).

Four different derivatisation temperature conditions were performed in the randomised order of 60, 85, 50 and 70°C. For each of the selected temperatures, four different reaction times in the order of 30, 60, 90 and 120 min was performed as shown in Table 3.3.

All batches consisted of only one condition (specific temperature and specific reaction time combination). Again, randomisation within conditions were not possible due to restrictive instrumentation availability and time limitation for this investigation. In order to avoid possible effect of prolonged waiting time on the instrument, a time schedule for the derivatisation was used to eliminate batches waiting to be injected on the GC-MS.

Table 3.3 Batch sequence design for adapted thermal derivatisation

	Adapted thermal derivatisation															
Temperature (°C)	60				85				50				70			
Reaction time (min)	30	60	90	120	30	60	90	120	30	60	90	120	30	60	90	120
Batch sequence	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
Replicates	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

3.4.3 Microwave-assisted derivatisation

For the microwave-assisted derivatisation, the dried organic acid standard mixture previously prepared (Section 3.2.3.1), was removed from the freezer and allowed to reach room temperature (25°C), followed by additional drying under a gentle stream of nitrogen at 37°C for 5 min. A volume of 50 µl pyridine, 50 µl BSTFA-TMCS (99:1%) and 50 µl C19:0Me (Section 3.2.3.2) were added to

each vial, whereafter the vials were capped, vortexed and microwaved using a Milestone ETHOS-easy microwave system (Magna Analytical, Johannesburg, South Africa) according to the energy and reaction time combination being tested (Table 3.4). After incubation the vials were allowed to cool for 10 min at room temperature, whereafter the samples were loaded for GC-MS analysis (Section 3.3.1).

Four different microwave energy settings were used for derivatisation in the randomised order of 230, 450, 150 and 350 W. For each of the selected energies, four different reaction times in the order of 1.5, 2.0, 3.0 and 4.0 min was tested as shown in Table 3.4.

All batches consisted of only one condition (specific energy and specific reaction time combination). Randomisation within conditions were not possible due to restrictive instrumentation availability and time limitation for this investigation. In order to avoid possible effect of prolonged waiting time on the instrument, a time schedule for the derivatisation was used to eliminate batches waiting to be injected on the GC-MS.

Table 3.4: Batch sequence design for microwave-assisted derivatisation

	Microwave-assisted derivatisation															
Microwave energy (W)	230				450				150				350			
Reaction time (min)	1.5	2.0	3.0	4.0	1.5	2.0	3.0	4.0	1.5	2.0	3.0	4.0	1.5	2.0	3.0	4.0
Batch sequence	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
Replicates	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

3.4.4 Adapted microwave-assisted derivatisation

The adapted microwave assisted derivatisation was performed with the organic acid mixture, prepared in advance (Section 3.2.3.1). The dried aliquots were removed from the freezer and allowed to reach room temperature, followed by drying under a gentle stream of nitrogen at 37°C for 5 min. Methoxymation was performed with the addition of 50 µl methoxyamine solution (Section 3.2.3.3) to the vials, capped and heated in an oven at 30°C for 90 min. The vials were allowed to cool down for 10 min at room temperature whereafter the silylation procedure followed. The silylation involved the addition of 50 µl BSTFA-TMCS (99:1%) and 50 µl C19:0Me internal standard (Section 3.2.3.2). The vials were capped, vortexed and microwaved according to the energy and reaction time combination being tested (Table 3.5). After incubation the vials were allowed to cool for 10 min and loaded for GC-MS analysis (Section 3.3.1).

Four different microwave energy settings were used for derivatisation in the randomised order of 230, 450, 150 and 350 W. For each of the selected energies, four different reaction times in the order of 1.5, 2.0, 3.0 and 4.0 min was tested as shown in Table 3.5.

All batches consisted of only one condition (specific energy and specific reaction time combination). Randomisation within conditions were not possible due to restrictive instrumentation availability and time limitation for this investigation. In order to avoid possible effect of prolonged waiting time on the instrument, a time schedule for the derivatisation was used to eliminate batches waiting to be injected on the GC-MS.

Table 3.5: Batch sequence design for adapted microwave-assisted derivatisation

	Adapted microwave-assisted derivatisation															
Microwave energy (W)	230				450				150				350			
Reaction time (min)	1.5	2.0	3.0	4.0	1.5	2.0	3.0	4.0	1.5	2.0	3.0	4.0	1.5	2.0	3.0	4.0
Batch sequence	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64
Replicates	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

3.5 Data processing

3.5.1 Data pre-processing

Data was pre-processed as recommended by Van den Berg *et al.* (2006). Non-derivatisation variation, amongst others, can be caused by volume loss due to vaporisation as a result of incubation at high temperatures with vial caps not sealing properly. The internal standard C19:0Me, which is not affected by derivatisation, was used to normalise the data. The relative responses were then used for further data analyses. Response values were acquired from quantitation reports and imported into Microsoft Office Excel 2016. The response for each derivative is proportional to the concentration of the derivative injected.

3.5.2 Data pre-treatment

After normalisation, two samples from two different batches were removed, because of an instrument error with a blocked needle. These two batches were processed with the remaining four replicates, instead of five replicates. The remaining data was firstly scaled through shifted log transformation, with the shift parameter set to one for the correction of deviation from normality. Secondly, autoscaling was also performed to equalise the importance of derivatives despite their different responses (Van den Berg *et al.*, 2006). Matrix laboratory (MATLAB) software were used to perform all data scaling pre-treatment methods.

3.5.3 Statistical analysis

After completion of the data pre-treatment, statistical analyses were performed as described and discussed in the relevant sections in Chapter 4. Multivariate outliers were determined by principal component analysis (PCA) score plots and Hotelling's T-squared (T^2) distance plots produced by the

PLS-Toolbox 8.2.1 (2016), Eigenvector Research software. Box-like plots were used to compare CV values between conditions for all derivatives within a dataset. The box-like plots were processed by a slightly altered version of the notBoxPlot.m MATLAB function. Microsoft Office Excel 2016 was further used to process scattered plots illustrating derivatisation efficiency for derivatives individually.

3.6 Overview of the experimental approach

The experimental approach outlined in Figure 3.2 was constructed in order to prepare and compare the predefined silylation derivatisation parameters required for thermal- and microwave-assisted derivatisation, as a prerequisite for GC-MS analysis of selected organic acids. The experimental approach was divided in five main steps, consisting of: 1) Compound selection, 2) Standard preparation, 3) GC-MS method standardisation, 4) Derivatisation parameter comparisons and 5) Statistical analysis.

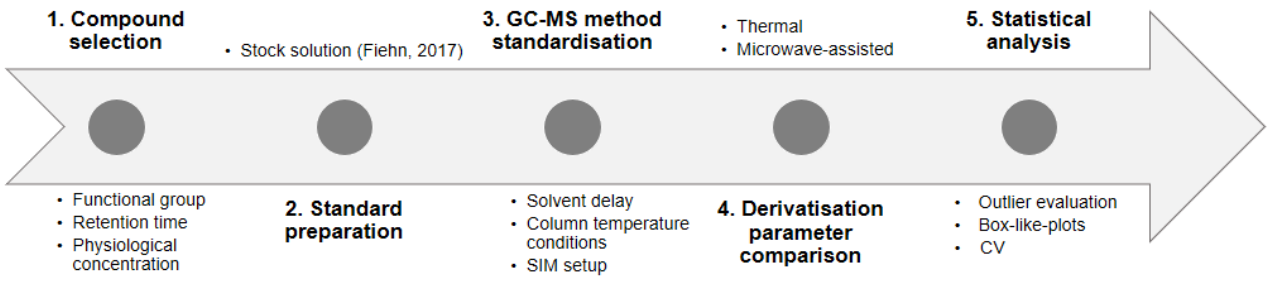


Figure 3.2: Global experimental approach. A global overview outline of the approach followed including: 1) Compound selection, 2) Standard preparation, 3) GC-MS method standardisation, 4) Derivatisation parameter comparison and 5) Statistical analysis.

In the first step of the experimental approach, organic acids were selected considering functional groups, physiological concentrations, chain length and the presence of aromatic ring structures (Table 2.3 and Table 2.4). Secondly a stock solution of all the organic acid standards was prepared and aliquoted according to the concentrations required for all future analysis (Section 3.2.3.1). After completing the standard preparation, a GC-MS method was standardised by adapting an in-house GC-MS method used at the NWU Metabolomics Platform (Section 3.3). Next, derivatisation parameter comparisons were performed (Section 3.4). In this project two experimental groups were compared with one another, the first being thermal derivatisation and the second microwave-assisted derivatisation.

For thermal derivatisation, the in-house used BSTFA-TMCS (99:1%) and pyridine were used to evaluate different temperature for different reaction times as experimental parameters (Fig. 3.3). Within each of these parameters, a range of four derivatisation temperatures (50, 60, 70 and 85°C) and four derivatisation times (30, 60, 90 and 120 min) were investigated in replicates of five. The

thermal derivatisation experiment also included an adapted method where the same derivatisation temperature and time parameters evaluated in the first set was repeated in replicates of five by using the same range of derivatisation temperatures and times, however prior to this derivatisation the addition of 20 mg of methylhydroxylamine hydrochloride per 1 ml pyridine heated in an oven at 30°C for 90 min was added to the sample.

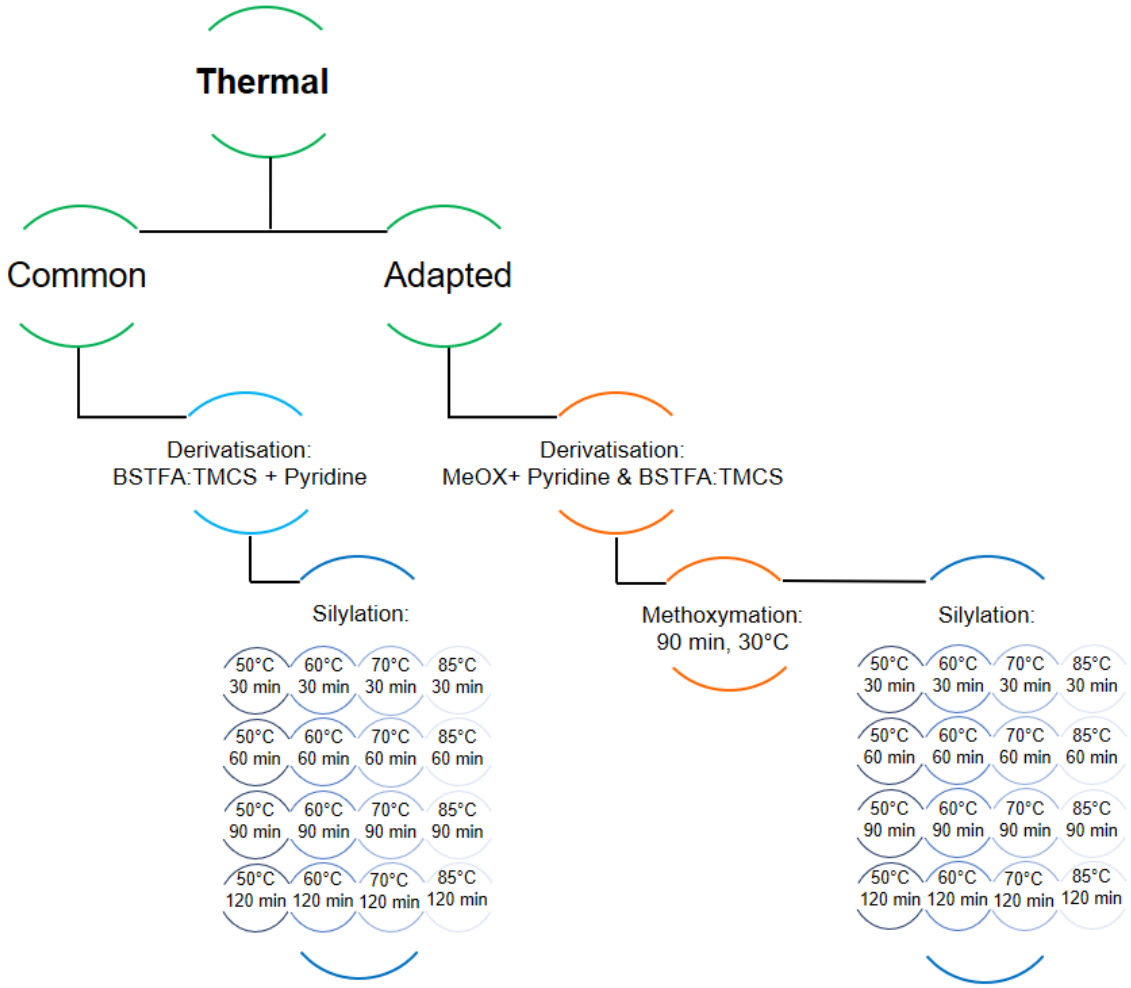


Figure 3.3: Thermal derivatisation procedure. A common and adapted thermal derivatisation method was used. Within each of these parameters a range of four derivatisation temperatures (50, 60, 70 and 85°C) and four derivatisation times (30, 60, 90 and 120 min) were investigated in replicates of five.

In the second experimental group of this study microwave-assisted derivatisation was applied (Fig. 3.4). For this group, the in-house used BSTFA-TMCS (99:1%) and pyridine silylation method was used to evaluate reaction time (min) and microwave energy (Watts) as experimental parameters. Within each of these parameters, a range of four derivatisation watts (150, 230, 350 and 450 W) and four derivatisation times (1.5, 2.0, 3.0 and 4.0 min) were investigated in replicates of five. The microwave-assisted derivatisation experiment also included an adapted method where the same derivatisation watt and reaction time parameters evaluated in the first set were repeated in replicates

of five using the same range of derivatisation watts and reaction times, once the addition of 20 mg of methylhydroxylamine hydrochloride per 1 ml pyridine heated in an oven at 30°C for 90 min was completed. All of the samples were analysed with the gas chromatography system coupled to a mass spectrometer, after silylation/ methoxymation was performed (Section 3.3.1).

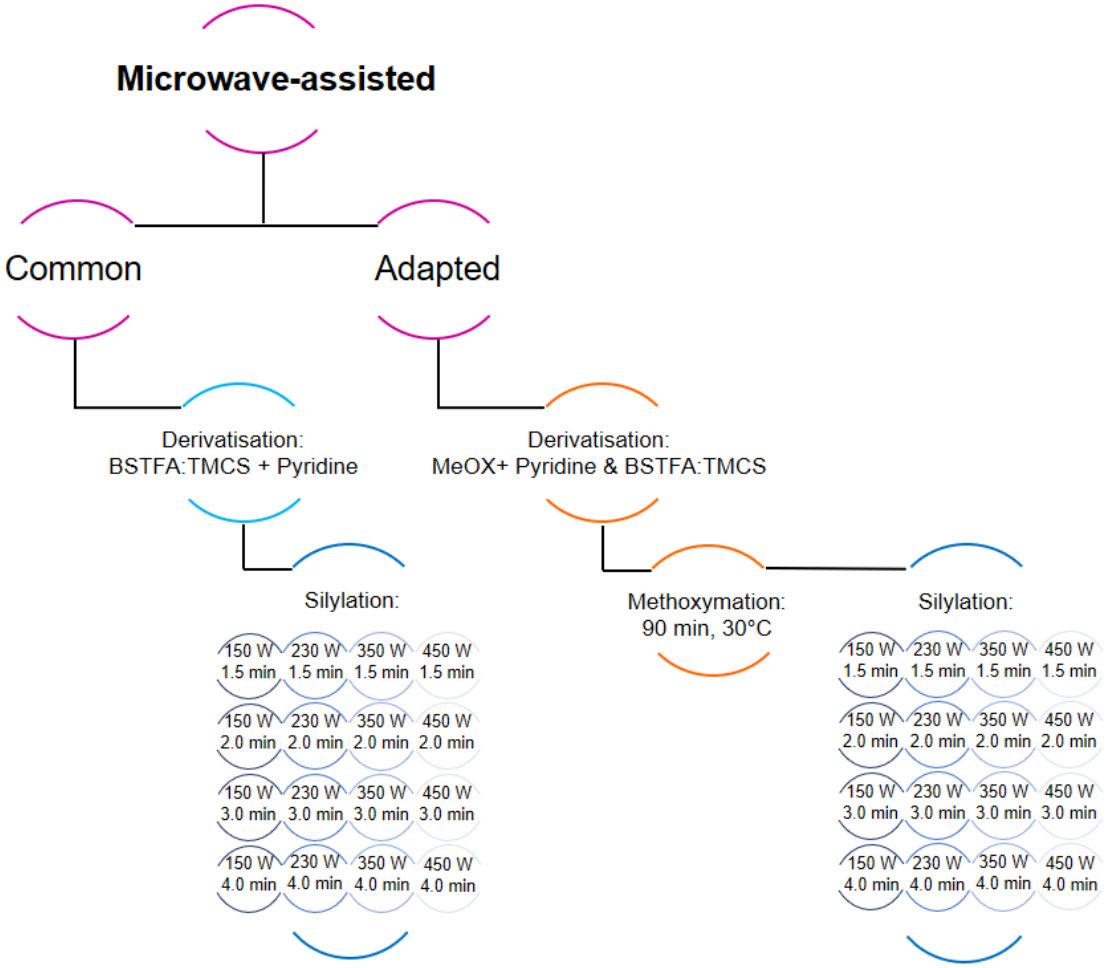


Figure 3.4: Microwave-assisted derivatisation procedure. A common and adapted microwave-assisted derivatisation method was used. Within each of these parameters a range of four derivatisation watts (150, 230, 350 and 450 W) and four derivatisation times (1.5, 2.0, 3.0 and 4.0 min) were investigated in replicates of five.

Lastly, data were retrieved and analysed as described in Section 3.5 and Chapter 4. The derivatisation parameters were compared according the performance criteria that included: relative response, repeatability, and derivatisation reaction time.

CHAPTER 4: RESULTS & DISCUSSION

4.1 Introduction

The aim of this investigation was to compare the effect of pre-defined derivatisation parameters (Section 3.4) required for gas chromatography (GC-MS) analysis of selected organic acids. This chapter presents and discusses the results as comparisons within and between the four derivatisation types (thermal, adapted thermal, microwave-assisted and adapted microwave-assisted). The results start with an overview of each derivatisation type, determining possible outliers within a batch (condition). Next, the repeatability of conditions within each derivatisation type will be compared and discussed. Compound specific comparison will follow to determine the effect of derivatisation on each individual compound with multiple derivatives considered. Lastly, an overview of sample derivatisation will be given focusing on reporduciblity of organic acids in one sample.

4.2 Experimental results

4.2.1 Thermal derivatisation

As described in Section 3.4.1 the thermal derivatisation experiments were performed at four pre-selected temperatures (50, 60, 70 and 85°C), each for four different reaction times (30, 60, 90 and 120 min) (Table 3.2). Each batch consisted of five replicates, except for the conditions at 85°C for 30 min and 85°C for 60 min where only four replicates were used as mentioned in Section 3.5.3.

4.2.1.1 Determination of outliers

Outliers can be seen as a sample, which deviates significantly from the average (Bro and Smilde, 2014), and has the power to indicate non-parametric variation caused by sample pre-treatment, storage, injection, detection or peak integration (Mastrangelo *et al.*, 2015). According to Mastrangelo *et al.* (2015), principal component analysis (PCA) and Hotelling's T-squared (T^2) are statistical approaches that can be used to determine outliers within GC-MS obtained data. The relative response dataset for thermal derivatisation was evaluated for the presence of outliers within the analysed replicates for all conditions. Outliers were determined by the two approaches: two-dimensional PCA using a 95% confidence interval and Hotelling's T^2. PCA provides a summarised projection of variation that is not due to the tested variables in a dataset (Johnson and Wichern, 2002) while Hotelling's T^2 measures the distance of scores derived from a PCA centroid scores plot (Sheriff *et al.*, 2018). Figure 4.1 illustrates the PCA scores plot (A) and Hotelling's T^2 distances plot (B) of five replicates for the thermal derivatisation condition of 50˚C at 30 min. Plots for the other conditions are given in Appendix A (Figs. A.1 - A.15).

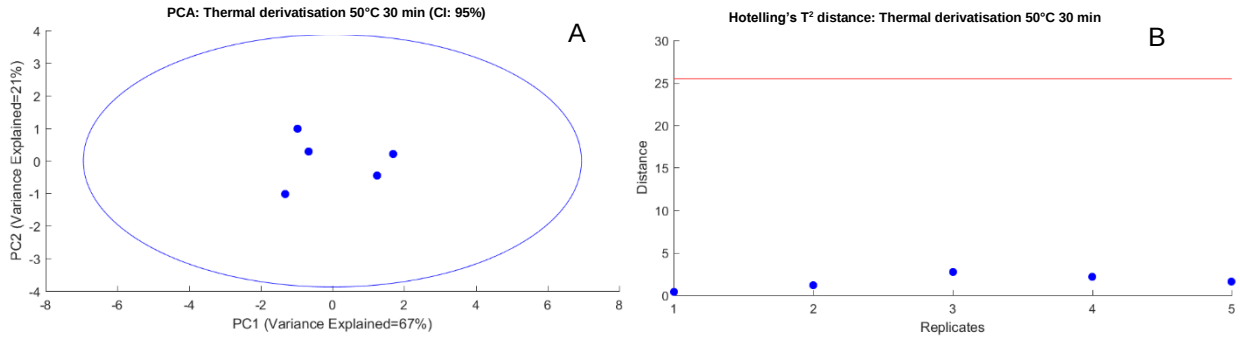


Figure 4.1: Outlier evaluation for thermal derivatisation at 50°C for 30 min. Principal component analysis (A) and Hotelling's T^2 (B) for five replicates in a batch.

The PCA scores plot in Figure 4.1A explains 88% of the total variance in the data, and in combination with the Hotelling's T^2 (Fig. 4.1B) establish that the five replicates are compositionally similar to each other. The results show that all the replicates fall within the 95% confidence interval making it possible to say that no outliers were present (Tugizimana *et al.*, 2016), for thermal derivatisation at 50°C for 30 min. For the PCA scores plot all replicates are grouped within the confidence interval (blue oval) and for the Hotellings T^2 result, all replicates display below the confidence threshold (red horizontal line). No outliers were present for any of the other thermal conditions evaluated (Appendix A, Figs. A.1 - A.15). Considering the absence of outliers within the thermal derivatisation conditions, all the replicates were used for further data analysis.

4.2.1.2 Comparison of within condition repeatability

In order to compare the repeatability within each condition (specific temperature and reaction time combination), coefficient of variance (CV) was calculated ($CV = \frac{standard\ deviation}{mean} x\ 100$) from the normalised data for replicates in each condition. CV is a statistical calculation used to characterise measurement variability (Parsons *et al.*, 2007) and is inversely proportional to repeatability (Moros *et al.*, 2017). An overview of the calculated CV (%) values for all conditions within the thermal derivatisation experiment are given in Figure 4.2.

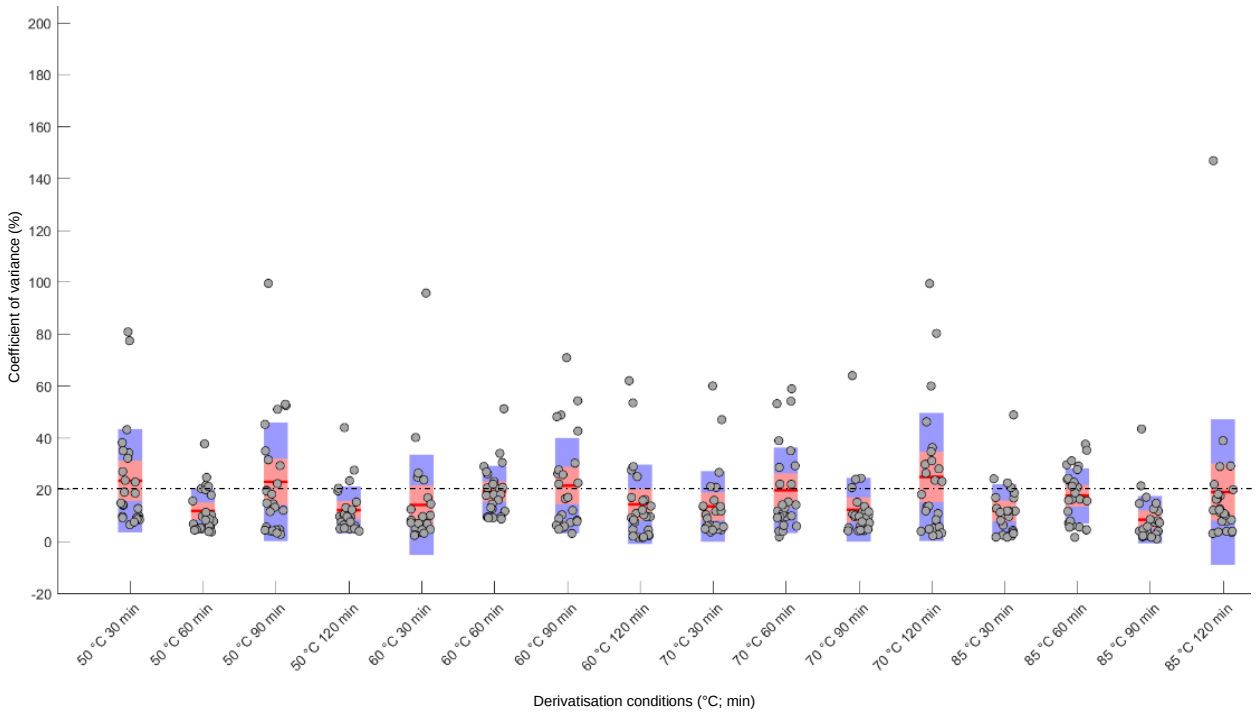


Figure 4.2: Coefficient of variation box-like plots for thermal derivatisation. Thermal derivatisation conditions are shown on the x-axis. At each condition the first value represent temperature (°C) and second value reaction time (min). Coefficient of variance (%) is shown on the y-axis. The black dash-dot line represents the CV ≤ 20% cut-off.

The term derivative refers to a derivatised compound (Bekele *et al.*, 2014). As discussed in Section 2.4.2, multiple derivatives may form for a compound. The box-like plots in Figure 4.2, summarises the average CV values (% - y-axis) for all detected derivatives at different thermal conditions investigated (˚C; min - x-axis). At each condition, the box-like plot shows the average CV (red central area), 95% confidence interval (pink middle area) and standard deviation (blue) for all detected derivatives (grey dots). For the purpose of evaluating repeatability, a CV cut-off value ≤ 20% (dash-dot line) was used as an acceptable value. For targeted analysis, the Food and Drug Administration (FDA) recommends a CV value of 15 to 20% (t'Kindt *et al.*, 2009). Sweetman (1991) consider errors in quantitative results < 50% as acceptable for diagnosis of inherited disorders, but errors < 20% for organic acids of clinical interest. For this study, derivatisation repeatability is considered as acceptable with coefficient variation ≤ 20%.

As presented in the box-like plots in Figure 4.2, it is clear that none of the investigated conditions resulted in repeatable CV values ≤ 20% for all 25 derivatives representing the 16 compounds at the same time. At this stage, 12 of the 16 conditions show an average CV ≤ 20%. The four conditions with average CV > 20%, include 50°C, 30 min; 50°C, 90 min; 60°C, 90 min and 70°C, 120 min. For compounds with more than one derivative, the CV may vary since derivatisation efficiency is dependent on temperature and reaction time (Christou *et al.*, 2014, Moros *et al.*, 2017). Thus, the

results for a condition may be skewed. Therefore, each individual derivative will be discussed separately in order of similar functional groups.

Tables 4.1 - 4.4 show the CV values calculated for each compound according to its associated derivative(s). Each table includes the four different reaction times for a specific temperature. The 25 derivatives included: Adipic acid 2TMS (Adi 2TMS); Cis-aconitic acid 3TMS (Aco 3TMS);Citric acid 3TMS (Cit 3TMS); Citric acid 4TMS (Cit 4TMS); Fumaric acid 2TMS (Fum 2TMS); Hippuric acid 1TMS (Hip 1TMS); Hippuric acid 2TMS (Hip 2TMS); 2-Ketoglutaric acid 1TMS (Ket 1TMS); 2-Ketoglutaric acid 2TMS (Ket 2TMS); 2-Ketoglutaric acid 3TMS (Ket 3TMS); Malonic acid 2TMS (Mal 2TMS); Malonic acid 3TMS (Mal 3TMS); N-acetyl-L-alanine 1TMS (Ace 1TMS); N-acetyl-L-alanine 2TMS (Ace 2TMS); Orotic acid 3TMS (Oro 3TMS); Oxalic acid 2TMS (Oxa 2TMS); Palmitic acid 1TMS (Pal 1TMS); Pentadecanoic acid 1TMS (Pen 1TMS); 3-Phenylbutyric acid 1TMS (Phe 1TMS); Pyruvic acid 2TMS (Pyr 2TMS); Sebacic acid 2TMS (Seb 2TMS); Succinylacetone 2TMS a (Suc 2TMS a); Succinylacetone 2TMS b (Suc 2TMS b); Succinylacetone 2TMS c (Suc 2TMS c) and Succinylacetone 3TMS (Suc 3TMS). These derivatives are sorted according to their increasing CV values within a condition. The bold line within each column is used to distinguish between CV values ≤ 20% (above the line) and > 20% (below the line).

Table 4.1: Coefficient of variance for thermal derivatives at 50°C

30 min	CV (%)	60 min	CV (%)	90 min	CV (%)	120 min	CV (%)
Adi 2TMS	6.44	Fum 2TMS	3.63	Adi 2TMS	2.71	Seb 2TMS	3.97
Pen 1TMS	7.47	Aco 3TMS	3.76	Pen 1TMS	3.21	Fum 2TMS	4.86
Seb 2TMS	8.31	Pen 1TMS	4.32	Fum 2TMS	3.95	Pal 1TMS	4.91
Pal 1TMS	8.48	Seb 2TMS	4.46	Pal 1TMS	4.10	Aco 3TMS	4.94
Cit 4TMS	8.95	Pal 1TMS	4.54	Seb 2TMS	4.32	Ace 1TMS	4.95
Ace 1TMS	9.08	Ket 3TMS	4.88	Aco 3TMS	4.42	Pen 1TMS	5.10
Fum 2TMS	9.13	Hip 1TMS	5.15	Ace 1TMS	4.49	Hip 1TMS	5.91
Ket 3TMS	9.57	Ket 2TMS	5.69	Hip 1TMS	5.51	Mal 2TMS	6.28
Hip 1TMS	9.74	Ace 1TMS	5.84	Mal 2TMS	11.44	Ket 3TMS	7.47
Aco 3TMS	12.65	Ace 2TMS	6.70	Suc 2TMS a	11.99	Oro 3TMS	7.51
Suc 2TMS a	13.87	Suc 2TMS c	7.76	Suc 2TMS c	13.16	Ace 2TMS	8.40
Suc 2TMS b	14.26	Adi 2TMS	8.24	Suc 2TMS b	14.44	Suc 2TMS c	9.43
Suc 2TMS c	14.78	Mal 2TMS	8.47	Ace 2TMS	14.75	Adi 2TMS	9.56
Mal 2TMS	18.59	Ket 1TMS	9.62	Phe 1TMS	18.21	Suc 2TMS a	9.63
Ket 1TMS	18.94	Suc 2TMS a	9.62	Mal 3TMS	19.40	Suc 2TMS b	9.98
Phe 1TMS	22.89	Suc 2TMS b	10.09	Suc 3TMS	19.71	Mal 3TMS	10.49
Ket 2TMS	23.52	Mal 3TMS	11.18	Oro 3TMS	22.25	Cit 4TMS	12.22
Cit 3TMS	26.88	Suc 3TMS	15.57	Hip 2TMS	29.19	Ket 1TMS	12.75
Mal 3TMS	32.07	Oro 3TMS	17.84	Ket 3TMS	31.46	Hip 2TMS	13.02
Suc 3TMS	34.17	Phe 1TMS	19.97	Ket 2TMS	34.88	Phe 1TMS	15.16
Oxa 2TMS	34.95	Hip 2TMS	20.34	Cit 3TMS	45.11	Suc 3TMS	19.44
Ace 2TMS	38.06	Oxa 2TMS	21.20	Oxa 2TMS	50.90	Ket 2TMS	20.50\
Hip 2TMS	43.04	Cit 4TMS	21.65	Ket 1TMS	52.34	Oxa 2TMS	23.36
Pyr 2TMS	77.34	Cit 3TMS	24.68	Pyr 2TMS	52.90	Cit 3TMS	27.46
Oro 3TMS	80.76	Pyr 2TMS	37.60	Cit 4TMS	99.44	Pyr 2TMS	43.87

Table 4.2: Coefficient of variance for thermal derivatives at 60°C

30 min	CV (%)	60 min	CV (%)	90 min	CV (%)	120 min	CV (%)
Pal 1TMS	2.33	Pal 1TMS	8.63	Pen 1TMS	3.05	Seb 2TMS	1.42
Ace 1TMS	2.48	Pen 1TMS	9.04	Pal 1TMS	4.68	Fum 2TMS	1.52
Pen 1TMS	3.15	Adi 2TMS	9.07	Fum 2TMS	5.34	Pal 1TMS	1.64
Fum 2TMS	3.49	Ace 1TMS	9.35	Seb 2TMS	5.78	Pen 1TMS	2.00
Suc 2TMS a	4.44	Fum 2TMS	9.38	Aco 3TMS	6.18	Ace 1TMS	2.27
Seb 2TMS	4.46	Seb 2TMS	9.39	Suc 2TMS c	7.19	Aco 3TMS	2.52
Aco 3TMS	4.58	Aco 3TMS	11.65	Suc 2TMS a	7.24	Ket 3TMS	4.37
Suc 2TMS c	4.97	Hip 1TMS	12.55	Phe 1TMS	7.69	Hip 1TMS	4.57
Suc 2TMS b	5.19	Phe 1TMS	12.97	Hip 1TMS	8.18	Phe 1TMS	7.54
Hip 1TMS	6.62	Cit 4TMS	14.41	Adi 2TMS	8.77	Adi 2TMS	8.29
Cit 4TMS	6.79	Ace 2TMS	15.92	Suc 2TMS b	10.20	Mal 2TMS	9.08
Phe 1TMS	6.80	Ket 3TMS	17.70	Ace 1TMS	11.94	Suc 2TMS c	9.50
Adi 2TMS	6.93	Suc 2TMS c	18.02	Suc 3TMS	16.44	Suc 2TMS a	9.55
Ket 3TMS	7.19	Mal 2TMS	18.17	Ket 3TMS	16.97	Suc 3TMS	10.21
Ket 1TMS	8.17	Suc 2TMS a	20.33	Oro 3TMS	22.11	Suc 2TMS b	10.78
Oxa 2TMS	9.59	Ket 2TMS	20.61	Mal 3TMS	22.53	Cit 4TMS	12.12
Mal 3TMS	10.06	Suc 2TMS b	20.85	Oxa 2TMS	25.72	Ace 2TMS	13.62
Mal 2TMS	12.38	Oxa 2TMS	22.06	Ket 2TMS	26.08	Oxa 2TMS	15.79
Oro 3TMS	14.45	Cit 3TMS	23.08	Ket 1TMS	27.54	Ket 1TMS	16.14
Pyr 2TMS	16.82	Mal 3TMS	26.04	Cit 4TMS	30.18	Ket 2TMS	16.94
Ace 2TMS	23.70	Oro 3TMS	26.78	Cit 3TMS	42.52	Mal 3TMS	24.98
Suc 3TMS	24.75	Ket 1TMS	28.89	Mal 2TMS	48.07	Cit 3TMS	27.48
Cit 3TMS	26.33	Pyr 2TMS	30.39	Pyr 2TMS	48.70	Oro 3TMS	28.85
Ket 2TMS	40.01	Suc 3TMS	33.94	Ace 2TMS	54.19	Hip 2TMS	53.37
Hip 2TMS	95.71	Hip 2TMS	51.14	Hip 2TMS	70.80	Pyr 2TMS	61.94

Table 4.3: Coefficient of variance for thermal derivatives at 70°C

30 min	CV (%)	60 min	CV (%)	90 min	CV (%)	120 min	CV (%)
Phe 1TMS	3.61	Hip 1TMS	1.78	Adi 2TMS	4.07	Pal 1TMS	2.18
Pal 1TMS	4.41	Pal 1TMS	3.85	Pen 1TMS	4.08	Seb 2TMS	2.56
Ket 2TMS	4.42	Seb 2TMS	3.89	Ace 1TMS	4.18	Fum 2TMS	3.24
Ace 1TMS	4.67	Suc 2TMS b	5.85	Fum 2TMS	4.22	Aco 3TMS	3.89
Pen 1TMS	4.83	Pen 1TMS	6.17	Pal 1TMS	4.30	Pen 1TMS	4.75
Ket 3TMS	5.05	Aco 3TMS	9.14	Seb 2TMS	4.61	Ket 1TMS	5.07
Adi 2TMS	5.37	Fum 2TMS	9.44	Aco 3TMS	5.18	Hip 1TMS	5.55
Seb 2TMS	5.66	Suc 2TMS a	9.54	Hip 1TMS	5.77	Suc 3TMS	8.34
Aco 3TMS	5.83	Suc 2TMS c	9.83	Ket 3TMS	7.13	Suc 2TMS c	10.72
Hip 1TMS	6.08	Ace 1TMS	10.57	Oro 3TMS	7.57	Adi 2TMS	11.70
Fum 2TMS	6.11	Mal 2TMS	11.73	Ket 1TMS	8.98	Suc 2TMS a	13.47
Cit 4TMS	6.22	Ace 2TMS	12.91	Mal 2TMS	9.46	Phe 1TMS	13.61
Ket 1TMS	8.83	Pyr 2TMS	13.40	Phe 1TMS	9.64	Suc 2TMS b	18.07
Suc 2TMS c	10.25	Adi 2TMS	14.07	Suc 2TMS c	9.97	Oxa 2TMS	23.09
Suc 2TMS a	11.67	Suc 3TMS	14.08	Cit 4TMS	10.08	Ace 1TMS	23.55
Suc 2TMS b	11.68	Phe 1TMS	15.22	Suc 2TMS a	10.53	Oro 3TMS	26.29
Mal 3TMS	13.44	Mal 3TMS	22.00	Ket 2TMS	10.92	Mal 3TMS	27.98
Mal 2TMS	13.52	Ket 1TMS	22.06	Suc 2TMS b	11.30	Ket 3TMS	29.69
Oro 3TMS	13.62	Cit 3TMS	28.51	Ace 2TMS	11.45	Mal 2TMS	31.06
Ace 2TMS	15.81	Ket 2TMS	29.12	Mal 3TMS	13.48	Hip 2TMS	34.59
Suc 3TMS	20.83	Oro 3TMS	34.95	Suc 3TMS	15.11	Ket 2TMS	36.16
Cit 3TMS	21.13	Oxa 2TMS	38.80	Oxa 2TMS	20.74	Cit 3TMS	46.10
Hip 2TMS	26.58	Ket 3TMS	53.09	Cit 3TMS	23.95	Ace 2TMS	59.90
Oxa 2TMS	46.91	Hip 2TMS	54.01	Hip 2TMS	24.38	Cit 4TMS	80.21
Pyr 2TMS	59.92	Cit 4TMS	58.82	Pyr 2TMS	63.89	Pyr 2TMS	99.37

Table 4.4: Coefficient of variance for thermal derivatives at 85°C

30 min	CV (%)	60 min	CV (%)	90 min	CV (%)	120 min	CV (%)
Seb 2TMS	1.62	Hip 1TMS	1.57	Pal 1TMS	0.98	Seb 2TMS	3.04
Pal 1TMS	1.65	Pal 1TMS	4.37	Pen 1TMS	1.61	Fum 2TMS	3.43
Adi 2TMS	2.06	Pen 1TMS	4.48	Aco 3TMS	1.78	Pal 1TMS	3.62
Fum 2TMS	2.24	Seb 2TMS	5.48	Fum 2TMS	2.14	Aco 3TMS	3.84
Ace 1TMS	2.41	Ace 1TMS	5.54	Seb 2TMS	2.17	Pen 1TMS	3.88
Aco 3TMS	3.66	Adi 2TMS	6.01	Ket 1TMS	2.31	Hip 1TMS	7.72
Hip 1TMS	4.26	Fum 2TMS	7.03	Ace 1TMS	2.75	Ket 3TMS	7.76
Ket 2TMS	6.23	Aco 3TMS	7.81	Ket 3TMS	2.89	Ace 1TMS	8.28
Pen 1TMS	7.05	Ace 2TMS	11.54	Ace 2TMS	3.69	Adi 2TMS	10.12
Ket 3TMS	8.00	Suc 2TMS c	15.59	Suc 2TMS b	3.90	Oro 3TMS	10.70
Suc 2TMS c	9.58	Cit 4TMS	15.86	Suc 2TMS c	4.00	Suc 2TMS a	10.90
Suc 2TMS a	11.17	Suc 2TMS b	16.17	Suc 2TMS a	4.05	Suc 2TMS c	11.19
Ace 2TMS	11.19	Suc 2TMS a	16.29	Hip 1TMS	4.56	Phe 1TMS	12.01
Phe 1TMS	11.22	Mal 2TMS	18.70	Adi 2TMS	5.74	Oxa 2TMS	12.20
Suc 2TMS b	11.71	Ket 3TMS	20.81	Oro 3TMS	7.19	Suc 2TMS b	12.58
Ket 1TMS	11.75	Phe 1TMS	21.28	Suc 3TMS	7.30	Mal 2TMS	16.22
Mal 2TMS	11.76	Hip 2TMS	22.80	Mal 2TMS	7.98	Ket 2TMS	16.32
Cit 3TMS	12.73	Suc 3TMS	23.84	Phe 1TMS	8.49	Suc 3TMS	17.59
Mal 3TMS	16.73	Ket 1TMS	24.33	Ket 2TMS	11.75	Cit 4TMS	18.22
Suc 3TMS	16.91	Oxa 2TMS	27.68	Mal 3TMS	12.49	Ket 1TMS	19.97
Cit 4TMS	18.69	Mal 3TMS	29.04	Hip 2TMS	14.58	Mal 3TMS	21.98
Oro 3TMS	20.72	Ket 2TMS	29.54	Oxa 2TMS	14.70	Ace 2TMS	28.85
Oxa 2TMS	22.52	Cit 3TMS	31.05	Cit 4TMS	17.03	Cit 3TMS	29.05
Hip 2TMS	24.13	Oro 3TMS	35.16	Cit 3TMS	21.42	Pyr 2TMS	38.78
Pyr 2TMS	48.75	Pyr 2TMS	37.46	Pyr 2TMS	43.37	Hip 2TMS	146.76

The first thermal derivatisation condition evaluated at 50°C for a reaction time of 30 min displayed 15 derivatives with a CV ≤ 20%; for 60 min, 20 derivatives; for 90 min, 16 derivatives and for 120 min, 21 derivatives resulted in a CV value ≤ 20% respectively (Table 4.1). In Table 4.2 the temperature investigated was 60°C. A reaction time of 30 min resulted in 20 derivatives with a CV ≤ 20%; for 60 min, 14 derivatives; for 90 min, 14 derivatives and for 120 min, 20 derivatives had a CV value ≤ 20% respectively. In Table 4.3 at a derivatisation temperature of 70°C, a reaction time of 30 min resulted in 20 derivatives with a CV ≤ 20%; for 60 min, 16 derivatives; for 90 min, 21 derivatives and for 120 min, 13 derivatives had a CV value ≤ 20% respectively. Lastly, the temperature investigated was 85°C where a reaction time of 30 min, displayed 21 derivatives with a CV ≤ 20%; for 60 min, 14 derivatives; for 90 min, 23 derivatives and for 120 min, 20 derivatives had a CV value ≤ 20% respectively (Table 4.4).

At 50°C, the largest number of derivatives had a CV ≤ 20% after derivatisation of 120 min. At 60°C, the same largest number of derivatives with a CV ≤ 20% were detected after 30 min and 120 min of derivatisation. At both 70°C and 85°C, the derivatisation time of 90 min resulted in the largest number of derivatives with a CV ≤ 20%. Overall the number of derivatives with a CV ≤ 20% greatly varies between the investigated temperatures and reaction times. According to literature, temperature and reaction time both have a significant effect on the derivatisation efficiency for organic acids as demonstrated by these results (Moros *et al.*, 2017).

Pentadecanoic acid is the compound used as internal standard influenced by derivatisation in this study. The derivative for pentadecanoic acid displayed CV < 10% indicating good repeatability for all thermal conditions. When derivatising pentadecanoic acid with BSTFA and hexane at 70°C for 30 min an intra-day repeatability of 0.4% was achieved (Yang *et al.*, 2009), however the current results show that lower CV values can be achieved at 85°C and 90 min when compared to the CV results obtained at 70°C for 30 min. Similar to pentadecanoic acid, the organic acids, palmitic acid and 3-phenylbutyric acid also consists of only one functional group namely a carboxyl group that can be derivatised (Table 2.3). 3-Phenylbutyric acid and palmitic acid do however differ with regards to chain length and the presence of an aromatic ring structure for 3-phenylbutyric acid. Tables 4.1 - 4.4 show palmitic acid 1TMS with CV < 10% for all conditions, but for 3-phenylbutyric acid CV < 23%. Based on the results of the tables above, 3-Phenylbutyric acid performs better at temperatures > 50°C with no prominent influence displayed by shorter/longer reaction times.

Adipic acid, fumaric acid, oxalic acid and sebacic acid are dicarboxylic acids with no additional functional groups. The results show that these dicarboxylic acids with no additional functional groups have not formed multiple derivatives for these compounds as suggested by Kouremenos *et al.* (2010a). The chain length of these organic acids differs with increasing length in the following order:

oxalic acid < fumaric acid < adipic acid < sebacic acid (Table 2.3). Tables 4.1 - 4.4 show that adipic acid 2TMS, fumaric acid 2TMS and sebacic acid 2TMS have CV values ≤ 20% at all conditions investigated. Adipic acid was previously reported with a low CV value (5%) following silylation at 75°C and 90 min (Pietrogrande *et al.*, 2010). Furthermore, the current findings correspond with a study that claims oxalic acid derivatises less efficiently than other dicarboxylic acids, since oxalic acid is a smaller derivatised acid (Docherty and Ziemann, 2001). In the current study, oxalic acid 2TMS shows CV values that range from 9.59 to 50.90% (Tables 4.1 - 4.4), with no influence by lower/ higher temperature or shorter/ longer reaction time. For cis-aconitic acid, a tricarboxylic acid, only one TMS derivative was detected, i.e. tri- TMS, corresponding to the fact that artefact formation is not a common characteristic of carboxylic acids (Little, 2014). Cis-aconitic acid 3TMS provided repeatable derivatisation with a CV ≤ 20% at all thermal conditions investigated (Tables 4.1 - 4.4).

Although orotic acid and pyruvic acid consist of keto- group(s), only one derivative was detected for each compound. Orotic acid is a monocarboxylic acid, but also contains two keto- groups, two amino groups and an aromatic ring structure (Table 2.3). A tri- TMS derivative of orotic acid was formed due to keto- enol tautomerisation of the two keto- and amino groups and carboxyl group that were silylated (Hušková *et al.*, 2004). CV vary greatly for this derivative with the highest CV (80.76%) obtained at 50°C for 30 min and the lowest CV (7.19%) at 85°C for 90 min. For the monocarboxylic acid, pyruvic acid 2TMS, a CV ≤ 20% was obtained at two conditions (at 60°C for 30 min and at 70°C for 60 min). In literature was found that pyruvic acid derivatised with BSTFA-TMCS (99:1%), resulted with the largest relative response factor when evaluating different derivatisation reagents at 70°C for 60 min, similar to the findings of this study, interestingly boron trifluoride/1-butanol resulted in the lowest relative response factor (Jurado-Sánchez *et al.*, 2012).

As indicated in Tables 4.1 - 4.4, more than one derivative was formed for the compounds: citric acid, hippuric acid, 2-ketoglutaric acid, malonic acid, N-acetyl-L-alanine and succinylacetone. The formation of multiple derivatives can be caused by insufficient or undesirable derivatisation conditions, steric hindrances or keto- groups that can (or cannot) be silylated in the same sample (Gerlo *et al.*, 2006, Little, 2014). Insufficient derivatisation may cause multiple derivatives for a compound, due to different TMS binding sites that silylate only partially.

As illustrated in Figure 4.3, N-acetyl-L-alanine consists of an amino and keto- group that can be either in an amide or imino-alcohol form. If the compound is in the imino-alcohol form, the hydroxyl group can be silylated, depending on the derivatisation condition (Gerlo *et al.*, 2006). N-acetyl-L-alanine also consists of a carboxyl group that can be silylated, thus resulting in a mono- or di- TMS derivative. In Tables 4.1 - 4.4 the CV for N-acetyl-L-alanine 1TMS is > 20% (23.55%) for only one condition (70°C; 120 min). N-acetyl-L-alanine 2TMS had a CV > 20% for 60°C, 30 min; 70°C, 120

min; 85°C, 30 min and 85°C, 90 min. Overall the results show that the N-acetyl-L-alanine 1TMS derivative had less variation and lower CV's than the di- TMS derivative.

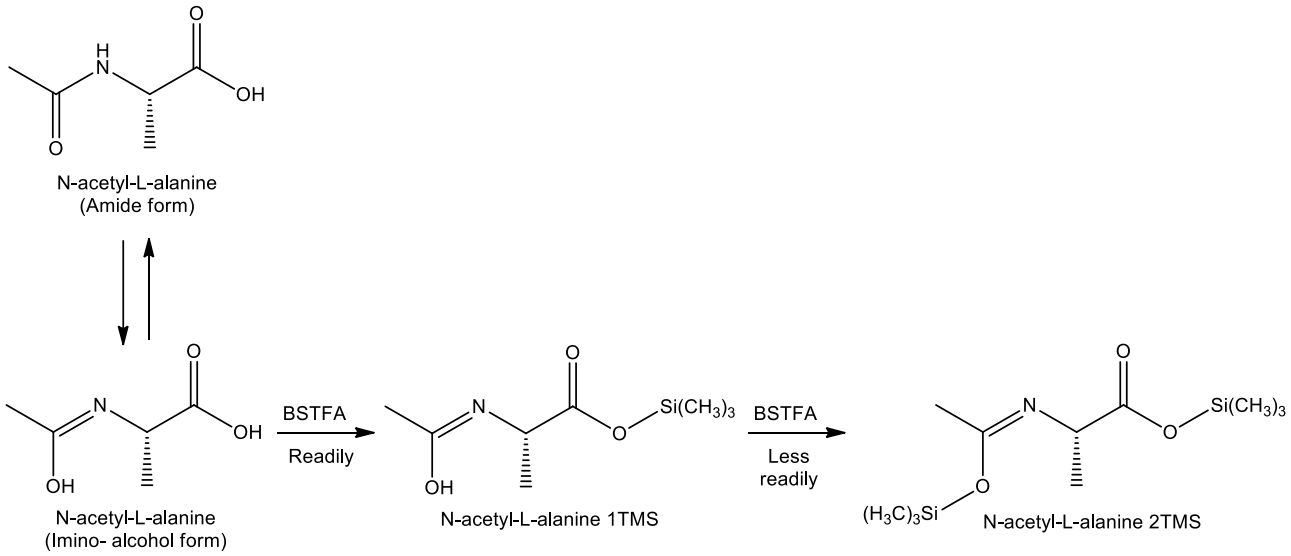


Figure 4.3: Formation of a mono- and di- TMS derivatives from N-acetyl-L-alanine. Derivatisation with BSTFA as reagent form easily a mono- TMS derivative and can also form a di- TMS derivative less readily.

Citric acid is another organic acid for which two derivatives were formed. Citric acid consists of four functional groups that can possibly be silylated (Table 2.3), however citric acid was only detected as a tri- TMS and tetra- TMS derivative (Tables 4.1 - 4.4). Formation of citric acid 3TMS is due to its alpha (α)- hydrogen that possibly lack space to become easily derivatised (Little, 2014). For citric acid 3TMS only one condition (70°C; 30 min) shows a CV ≤ 20%, but even though four of the other conditions have CV values > 20%, it still shows less variation than citric acid 4TMS (Tables 4.1 - 4.4). It is known that multiple derivatives form multiple peaks with lower peak intensities on a chromatogram that may complicate compound quantification (Little, 2014). Small peak areas result in low signal-to-noise ratios which means that the peak integration is greatly affected by the baseline (Amigo *et al.*, 2008). This may result in variation in integration, explaining the high CV for some of the multiple derivatives of this study, such as citric acid 3TMS and hippuric acid 2TMS.

Tables 4.1 - 4.4 display two derivatives for hippuric acid. The mono- TMS derivative of hippuric acid has a CV ≤ 20% for all conditions, but the di- TMS only has a CV ≤ 20% for the two conditions 60°C for 120 min and 70°C for 90 min. Hippuric acid consists of an aromatic ring structure, one keto-, one amino- and one carboxyl group (Table 2.3). Similar to N-acetyl-L-alanine, hippuric acid can also be either in its amide or imino- alcohol form. If the compound is in the imino- alcohol form, the hydroxyl group can be silylated, depending on the derivatisation condition. Hippuric acid also consist of a carboxyl group that can be silylated thus resulting in mono- or di- TMS derivatives, depending on the derivatisation condition (Wachsmuth *et al.*, 2015).

The compound 2-ketoglutaric acid resulted in three derivatives for all conditions (Tables 4.1 - 4.4), two carboxyl groups and one keto- group that can be silylated when looking at 2-ketoglutaric acid. As discussed in Section 2.4.3.1 a keto- group can form multiple derivatives due to keto- enol tautomerisation that occasionally occur (Qiu and Reed, 2014), explaining the formation of the di- TMS- and tri- TMS derivatives in the results (Tables 4.1 - 4.4). All three derivatives were detected at all thermal derivatisation conditions. In Tables 4.1 - 4.4 CV values for 2-ketoglutaric acid 1TMS vary from 2.31 to 52.34%, for 2-ketoglutaric acid 2TMS CVs range from 4.42 to 40.01% and for 2-ketoglutaric acid 3TMS from 2.89 to 53.09%. At most conditions 2-ketoglutaric acid 3TMS showed less variation than the mono- and di- TMS derivatives.

Malonic acid has a similar chemical structure than fumaric acid. Malonic acid has two functional groups that can substitute active hydrogens for silyl groups (Table 2.3). However, a derivative for malonic acid with three TMS groups and a derivative with two TMS groups were present (Tables 4.1 - 4.4). The tri- TMS derivative formed due to further silylation of the di- TMS derivative through enolisation of one of the ester groups (Mamer and Tjoa, 1973, Kouremenos *et al.*, 2010a), since malonic acid has a shorter chain length. The CV values for the di- TMS derivative of malonic acid ranged from 6.28 to 48.07% with two conditions having CV values above 20% (at 60°C for 90 min and at 70°C for 120 min). The CV values for the tri- TMS derivative of malonic acid ranged from 10.06 to 32.07%, but with eight conditions above 20%. Four conditions of the tri- TMS derivative provide lower CV values than the di- TMS derivative. A study investigating malonic acid in atmospheric aerosols showed a CV value of 6% for this compound following triplicate analysis and derivatisation with BSTFA-TMCS (99:1%) at 75°C for 90 min (Pietrogrande *et al.*, 2010), which was not a condition used for evaluation in the current study. All together the results displayed in Tables 4.1 - 4.4 indicate that neither temperature nor reaction time influences the derivatisation efficiency between the two derivatives of malonic acid.

The chemical structure of succinylacetone consists of two keto- groups and one carboxyl group (Table 2.3). Four derivatives for succinylacetone including a tri- TMS derivative and three di- TMS structural isomers were detected. Almost all thermal conditions provided a CV ≤ 20% for the three di- TMS derivatives, except 60°C for 60 min for the isomers succinylacetone 2TMS a and b. The CV for succinylacetone 3TMS vary between 7.30 to 34.17%, with a CV for five conditions above 20%. Considering the drawbacks associated with obtaining multiple derivatives it is recommended to select derivatisation conditions which minimise the incidence of multiple derivatives (Bekele *et al.*, 2014). Although results displayed that some conditions provided more repeatable derivatisation, the current investigated conditions failed to eliminate formation of multiple derivatives for succinylacetone amongst other compounds.

4.2.2 Adapted thermal derivatisation

As described in Section 3.4.2 the adapted thermal derivatisation experiments were performed at four different temperatures (50, 60, 70 and 85°C), each for four different reaction times (30, 60, 90 and 120 min) (Table 3.3). Each batch consisting of five replicates.

4.2.2.1 Determination of outliers

The presence of outliers within the analysed replicates for all conditions evaluated (Section 4.2.1.1) were investigated using the relative response dataset following adapted thermal derivatisation. In Figure 4.4 the PCA scores plot (A) and Hotelling's T^2 distance plot (B) of five replicates for the adapted thermal condition, 50˚C; 30 min are given. The other adapted thermal conditions evaluated are given in the same format in Appendix A (Figs. A.16 - A.30).

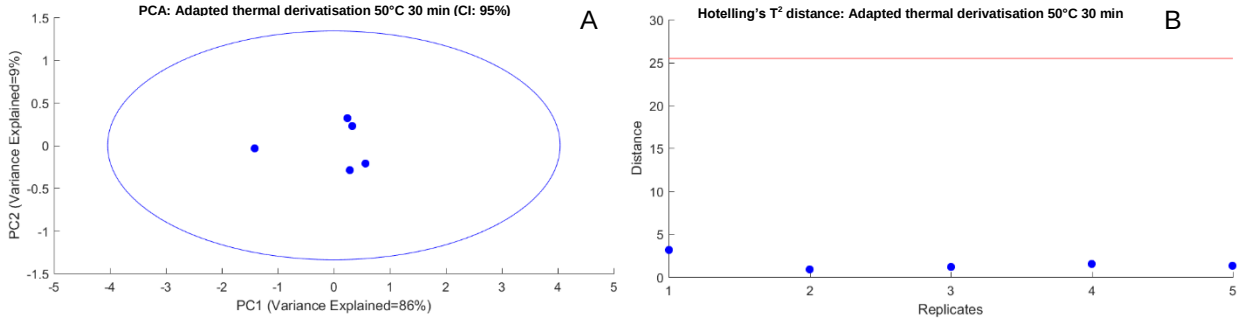


Figure 4.4: Outlier evaluation for adapted thermal derivatisation at 50°C for 30 min. Principal component analysis (A) and Hotelling's T^2 (B) for five replicates in a batch.

The data contained in the PCA scores plot (Fig. 4.4A) and Hotelling's T^2 distance plot (Fig. 4.4B) illustrate that the samples are compositionally similar to each other as can be expected when preparing and analysing the same sample in five replicates. As in Section 4.2.1.1, no outliers were present for this adapted thermal derivatisation condition, when considering the 95% confidence interval and 95% confidence threshold applied to the data. In Appendix A, Figures A.16 - A.30 confirm the absence of outliers, making it possible to use all of the data obtained from these experiments in the succeeding results.

4.2.2.2 Comparison of within condition repeatability

In accordance with Section 4.2.1.2 the repeatability within each adapted thermal condition (specific temperature and reaction time combination) was compared by calculating the CV. An overview of the calculated CV (%) values for all conditions within the adapted thermal derivatisation experiment are given in Figure 4.5.

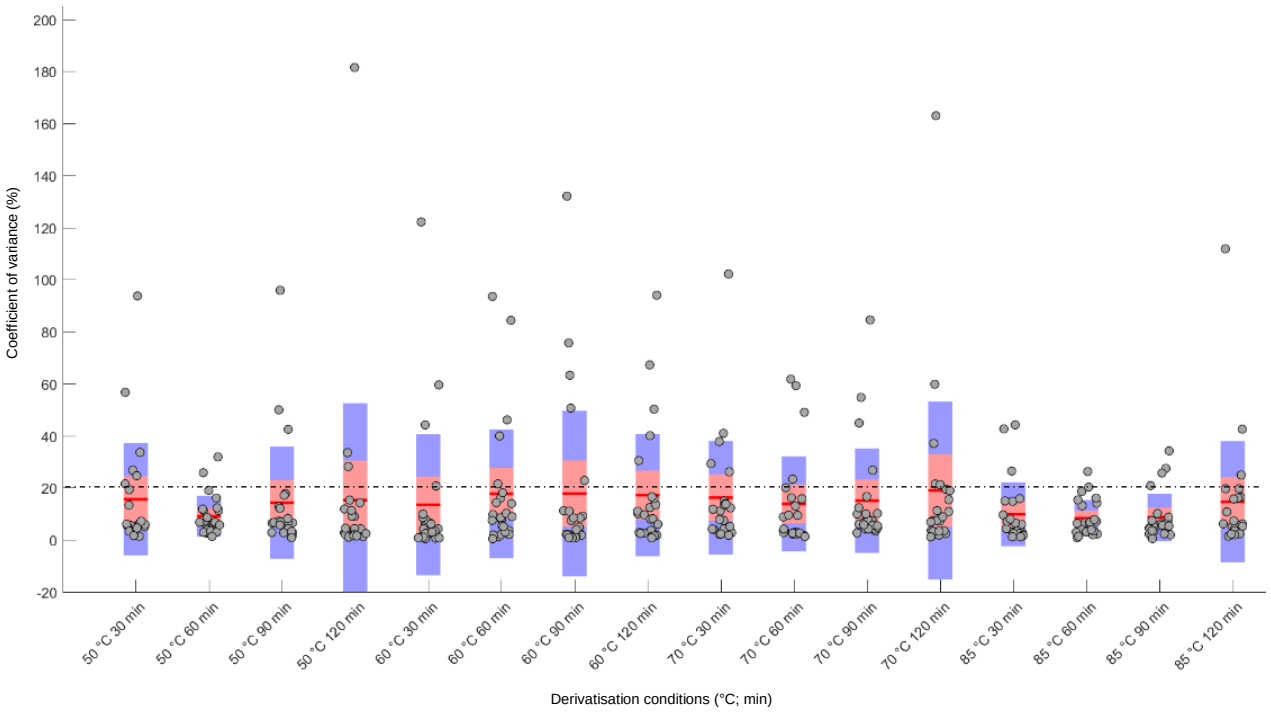


Figure 4.5: Box-like plots of conditions evaluated for adapted thermal derivatisation. Adapted thermal derivatisation conditions are shown on the x-axis. At each condition the first value represent temperature (°C) and second value reaction time (min). Coefficient of variance (%) is shown on the y-axis. The black dash-dot line represents the CV ≤ 20% cut-off.

As presented in the box-like-plots in Figure 4.5, it is clear that none of the investigated conditions resulted in repeatable CV values (≤ 20%) for all 24 derivatives representing the 16 compounds. Only, 12 of the 16 conditions show an average CV ≤ 20%. The four conditions with average CV > 20%, include 70°C, 30 min; 70°C, 60 min; 70°C, 90 min and 70°C, 120 min. As compound derivatisation efficiency is still dependent on temperature and time within the adapted thermal derivatisation condition, the succeeding discussion will focus on derivatives separately.

Tables 4.5 - 4.8 show the CV values calculated for each compound according to its associated derivative(s). Since multiple derivatives for a compound are typically associated with complicated compound quantitation (Little, 2014), methoxymation was recommended to stabilise keto- groups in order to obtain less derivatives for a compound (Lai and Fiehn, 2016). Results show that only 2-ketoglutaric acid formed one less derivative when the compound was methoxymated (M), when compared to the results without the use of methoxymation. Tables 4.5 - 4.8, consist of four different reaction times for a specific temperature, displaying the 24 derivatives analysed including: Adipic acid 2TMS (Adi 2TMS); Cis-aconitic acid 3TMS (Aco 3TMS); Citric acid 3TMS (Cit 3TMS); Citric acid 4TMS (Cit 4TMS); Fumaric acid 2TMS (Fum 2TMS); Hippuric acid 1TMS (Hip 1TMS); Hippuric acid 2TMS (Hip 2TMS); 2-Ketoglutaric acid 1M 2TMS a (Ket 1M 2TMS a); 2-Ketoglutaric acid 1M 2TMS b (Ket 1M 2TMS b); Malonic acid 2TMS (Mal 2TMS); Malonic acid 3TMS (Mal 3TMS); N-acetyl-L-alanine 1TMS (Ace 1TMS); N-acetyl-L-alanine 2TMS (Ace 2TMS); Orotic acid 3TMS (Oro 3TMS);

Oxalic acid 2TMS (Oxa 2TMS); Palmitic acid 1TMS (Pal 1TMS); Pentadecanoic acid 1TMS (Pen 1TMS); 3-Phenylbutyric acid 1TMS (Phe 1TMS); Pyruvic acid 1M 1TMS (Pyr 1M 1TMS); Sebacic acid 2TMS (Seb 2TMS); Succinylacetone 1M 1TMS a (Suc 1M 1TMS a); Succinylacetone 1M 1TMS b (Suc 1M 1TMS b); Succinylacetone 1M 1TMS c (Suc 1M 1TMS c) and Succinylacetone 1M 1TMS d (Suc 1M 1TMS d). Again, the derivatives are sorted in order of increasing CV values within a condition and the bold line indicates the 20% CV cut-off value.

Table 4.5: Coefficient of variance for adapted thermal derivatives at 50°C

30 min	CV (%)	60 min	CV (%)	90 min	CV (%)	120 min	CV (%)
Cit 4TMS	1.39	Pal 1TMS	1.29	Pal 1TMS	0.85	Fum 2TMS	1.07
Pal 1TMS	1.63	Fum 2TMS	2.53	Aco 3TMS	1.41	Ace 1TMS	1.30
Adi 2TMS	3.31	Ace 1TMS	2.57	Fum 2TMS	2.74	Seb 2TMS	1.52
Ace 1TMS	3.43	Adi 2TMS	2.89	Ace 1TMS	2.78	Aco 3TMS	1.57
Fum 2TMS	3.52	Seb 2TMS	3.08	Adi 2TMS	2.84	Ket 1M 2TMS a	2.14
Seb 2TMS	4.34	Aco 3TMS	3.44	Seb 2TMS	3.21	Adi 2TMS	2.14
Pen 1TMS	4.56	Cit 4TMS	3.84	Ket 1M 2TMS b	4.29	Pal 1TMS	2.43
Suc 1M 1TMS d	5.01	Ket 1M 2TMS a	4.41	Oro 3TMS	5.72	Suc 1M 1TMS b	2.52
Suc 1M 1TMS b	5.16	Ket 1M 2TMS b	4.55	Ket 1M 2TMS a	6.43	Suc 1M 1TMS c	2.71
Suc 1M 1TMS c	5.49	Pen 1TMS	5.75	Suc 1M 1TMS b	6.58	Suc 1M 1TMS d	2.73
Ket 1M 2TMS b	5.51	Suc 1M 1TMS b	6.53	Suc 1M 1TMS d	6.66	Ket 1M 2TMS b	3.85
Mal 2TMS	5.87	Suc 1M 1TMS c	6.57	Mal 3TMS	6.71	Suc 1M 1TMS a	4.29
Ket 1M 2TMS a	6.07	Suc 1M 1TMS a	6.80	Suc 1M 1TMS c	6.86	Mal 2TMS	4.34
Suc 1M 1TMS a	6.42	Suc 1M 1TMS d	6.87	Cit 4TMS	6.91	Cit 4TMS	4.48
Aco 3TMS	7.14	Mal 2TMS	8.60	Suc 1M 1TMS a	7.21	Phe 1TMS	8.98
Hip 1TMS	13.33	Mal 3TMS	11.31	Pen 1TMS	8.21	Oro 3TMS	9.13
Phe 1TMS	19.29	Phe 1TMS	11.35	Hip 1TMS	12.15	Oxa 2TMS	11.32
Pyr 1M 1TMS	21.49	Oro 3TMS	11.84	Mal 2TMS	12.77	Mal 3TMS	11.87
Oxa 2TMS	24.66	Hip 1TMS	12.30	Oxa 2TMS	17.14	Pen 1TMS	14.26
Mal 3TMS	26.79	Oxa 2TMS	16.12	Phe 1TMS	17.78	Cit 3TMS	15.26
Oro 3TMS	33.60	Cit 3TMS	18.99	Cit 3TMS	42.44	Pyr 1M 1TMS	28.20
Cit 3TMS	56.70	Ace 2TMS	25.81	Pyr 1M 1TMS	49.96	Hip 1TMS	33.53
Ace 2TMS	93.70	Pyr 1M 1TMS	31.86	Ace 2TMS	95.86	Ace 2TMS	181.46
Hip 2TMS	N/D	Hip 2TMS	N/D	Hip 2TMS	N/D	Hip 2TMS	N/D

Table 4.6: Coefficient of variance for adapted thermal derivatives at 60°C

30 min	CV (%)	60 min	CV	90 min	CV (%)	120 min	CV
Adi 2TMS	0.49	Pal 1TMS	0.43	Seb 2TMS	0.71	Pal 1TMS	0.79
Fum 2TMS	0.62	Ace 1TMS	1.22	Aco 3TMS	0.88	Fum 2TMS	1.43
Seb 2TMS	0.74	Adi 2TMS	1.86	Pal 1TMS	0.91	Seb 2TMS	1.56
Pal 1TMS	0.75	Fum 2TMS	2.01	Ace 1TMS	1.39	Adi 2TMS	1.82
Aco 3TMS	1.03	Aco 3TMS	2.12	Fum 2TMS	1.67	Pen 1TMS	2.47
Ace 1TMS	1.09	Seb 2TMS	2.93	Ket 1M 2TMS a	1.81	Aco 3TMS	2.65
Ket 1M 2TMS b	1.71	Hip 1TMS	3.59	Suc 1M 1TMS d	1.84	Ace 1TMS	2.68
Ket 1M 2TMS a	2.25	Pen 1TMS	5.10	Adi 2TMS	1.88	Ket 1M 2TMS a	2.84
Oro 3TMS	2.45	Ket 1M 2TMS a	5.55	Suc 1M 1TMS c	2.01	Hip 1TMS	3.66
Pen 1TMS	3.01	Oro 3TMS	7.48	Suc 1M 1TMS b	2.26	Oro 3TMS	5.98
Phe 1TMS	3.42	Suc 1M 1TMS b	7.78	Suc 1M 1TMS a	3.05	Mal 2TMS	7.96
Cit 4TMS	4.23	Suc 1M 1TMS d	8.51	Pen 1TMS	3.97	Cit 4TMS	8.14
Hip 1TMS	4.26	Mal 2TMS	8.91	Oro 3TMS	4.96	Ket 1M 2TMS b	9.63
Suc 1M 1TMS b	5.95	Suc 1M 1TMS c	9.68	Mal 2TMS	7.45	Suc 1M 1TMS b	10.08
Mal 2TMS	6.09	Suc 1M 1TMS a	10.19	Hip 1TMS	8.42	Suc 1M 1TMS d	11.00
Suc 1M 1TMS d	6.13	Ket 1M 2TMS b	13.93	Phe 1TMS	8.44	Suc 1M 1TMS c	12.45
Suc 1M 1TMS c	6.54	Phe 1TMS	14.32	Ket 1M 2TMS b	9.15	Suc 1M 1TMS a	13.49
Suc 1M 1TMS a	7.93	Mal 3TMS	16.14	Oxa 2TMS	11.00	Mal 3TMS	15.86
Cit 3TMS	8.56	Cit 4TMS	18.14	Cit 4TMS	11.21	Phe 1TMS	16.56
Oxa 2TMS	9.90	Oxa 2TMS	21.51	Mal 3TMS	22.79	Oxa 2TMS	30.44
Mal 3TMS	20.71	Hip 2TMS	39.90	Cit 3TMS	50.60	Cit 3TMS	40.04
Pyr 1M 1TMS	44.17	Cit 3TMS	46.09	Hip 2TMS	63.24	Ace 2TMS	50.19
Hip 2TMS	59.53	Pyr 1M 1TMS	84.32	Pyr 1M 1TMS	75.65	Pyr 1M 1TMS	67.20
Ace 2TMS	122.14	Ace 2TMS	93.54	Ace 2TMS	132.05	Hip 2TMS	93.99

Table 4.7: Coefficient of variance for adapted thermal derivatives at 70°C

30 min	CV (%)	60 min	CV (%)	90 min	CV (%)	120 min	CV
Pal 1TMS	1.76	Pal 1TMS	1.27	Pal 1TMS	2.66	Pal 1TMS	1.17
Ace 1TMS	2.08	Suc 1M 1TMS d	1.68	Suc 1M 1TMS b	3.35	Fum 2TMS	1.75
Aco 3TMS	2.29	Ace 1TMS	2.07	Ace 1TMS	4.04	Adi 2TMS	1.84
Adi 2TMS	2.77	Suc 1M 1TMS c	2.16	Aco 3TMS	4.20	Ace 1TMS	2.44
Cit 4TMS	3.37	Aco 3TMS	2.56	Adi 2TMS	4.25	Aco 3TMS	3.25
Ket 1M 2TMS b	3.93	Seb 2TMS	2.63	Fum 2TMS	4.70	Seb 2TMS	3.31
Seb 2TMS	4.21	Hip 1TMS	2.79	Seb 2TMS	5.40	Cit 4TMS	3.55
Fum 2TMS	4.22	Adi 2TMS	2.81	Oro 3TMS	5.62	Ket 1M 2TMS a	3.66
Pen 1TMS	5.13	Fum 2TMS	2.93	Pen 1TMS	5.80	Ket 1M 2TMS b	6.89
Ket 1M 2TMS a	7.44	Suc 1M 1TMS a	3.40	Cit 4TMS	6.05	Suc 1M 1TMS b	7.19
Mal 2TMS	7.81	Ket 1M 2TMS a	3.85	Suc 1M 1TMS d	6.63	Suc 1M 1TMS d	7.48
Suc 1M 1TMS b	11.12	Oro 3TMS	4.17	Suc 1M 1TMS a	7.38	Suc 1M 1TMS c	7.49
Suc 1M 1TMS d	11.79	Mal 3TMS	8.78	Ket 1M 2TMS a	7.65	Mal 2TMS	8.85
Suc 1M 1TMS c	12.31	Suc 1M 1TMS b	9.43	Ket 1M 2TMS b	8.43	Suc 1M 1TMS a	10.88
Suc 1M 1TMS a	13.40	Pen 1TMS	9.52	Suc 1M 1TMS c	9.92	Oro 3TMS	11.23
Oro 3TMS	13.73	Mal 2TMS	13.02	Hip 1TMS	9.97	Pen 1TMS	15.41
Hip 1TMS	14.00	Phe 1TMS	15.78	Phe 1TMS	10.16	Phe 1TMS	18.91
Mal 3TMS	14.97	Ket 1M 2TMS b	16.18	Mal 2TMS	12.36	Hip 1TMS	19.43
Phe 1TMS	26.16	Cit 4TMS	20.06	Mal 3TMS	16.64	Oxa 2TMS	21.23
Oxa 2TMS	29.28	Oxa 2TMS	23.32	Oxa 2TMS	26.77	Mal 3TMS	21.47
Cit 3TMS	37.82	Cit 3TMS	49.01	Cit 3TMS	44.86	Pyr 1M 1TMS	37.06
Pyr 1M 1TMS	40.96	Pyr 1M 1TMS	59.24	Pyr 1M 1TMS	54.73	Cit 3TMS	59.79
Ace 2TMS	102.13	Ace 2TMS	61.73	Ace 2TMS	84.51	Ace 2TMS	162.9
Hip 2TMS	N/D	Hip 2TMS	N/D	Hip 2TMS	N/D	Hip 2TMS	N/D

Table 4.8: Coefficient of variance for adapted thermal derivatives at 85°C

30 min	CV	60 min	CV (%)	90 min	CV (%)	120 min	CV (%)
Pal 1TMS	1.19	Cit 4TMS	0.83	Pal 1TMS	0.49	Ket 1M 2TMS a	1.45
Seb 2TMS	1.20	Pal 1TMS	1.38	Aco 3TMS	1.83	Seb 2TMS	1.96
Aco 3TMS	1.71	Adi 2TMS	1.91	Fum 2TMS	1.94	Pal 1TMS	2.18
Fum 2TMS	1.83	Fum 2TMS	2.28	Adi 2TMS	2.27	Aco 3TMS	2.37
Ace 1TMS	2.46	Seb 2TMS	2.93	Seb 2TMS	2.34	Fum 2TMS	3.42
Adi 2TMS	2.47	Ace 1TMS	3.13	Ace 1TMS	3.34	Adi 2TMS	3.51
Suc 1M 1TMS c	2.60	Ket 1M 2TMS a	3.21	Pen 1TMS	3.58	Ace 1TMS	4.24
Suc 1M 1TMS b	2.91	Aco 3TMS	3.39	Ket 1M 2TMS a	4.17	Suc 1M 1TMS a	4.77
Suc 1M 1TMS d	2.95	Pen 1TMS	3.84	Ket 1M 2TMS b	4.53	Suc 1M 1TMS b	5.06
Ket 1M 2TMS a	3.38	Ket 1M 2TMS b	4.00	Suc 1M 1TMS b	5.06	Cit 4TMS	5.21
Suc 1M 1TMS a	3.42	Hip 1TMS	6.33	Suc 1M 1TMS d	5.29	Suc 1M 1TMS d	5.56
Cit 4TMS	4.32	Suc 1M 1TMS c	6.45	Cit 4TMS	5.31	Suc 1M 1TMS c	6.12
Ket 1M 2TMS b	5.95	Suc 1M 1TMS b	6.61	Suc 1M 1TMS c	5.42	Ket 1M 2TMS b	6.18
Phe 1TMS	6.52	Suc 1M 1TMS a	6.77	Cit 3TMS	5.63	Pen 1TMS	7.28
Oro 3TMS	7.12	Suc 1M 1TMS d	7.12	Suc 1M 1TMS a	6.23	Oro 3TMS	10.76
Oxa 2TMS	8.00	Oro 3TMS	7.80	Hip 1TMS	7.38	Phe 1TMS	15.72
Cit 3TMS	9.60	Mal 3TMS	13.14	Phe 1TMS	7.45	Cit 3TMS	16.12
Hip 1TMS	14.81	Mal 2TMS	14.08	Oro 3TMS	8.73	Mal 2TMS	16.40
Mal 2TMS	14.96	Cit 3TMS	15.37	Mal 2TMS	10.11	Hip 1TMS	19.63
Pen 1TMS	15.82	Oxa 2TMS	16.07	Oxa 2TMS	20.85	Oxa 2TMS	19.64
Mal 3TMS	26.44	Ace 2TMS	18.58	Ace 2TMS	25.68	Mal 3TMS	24.90
Pyr 1M 1TMS	42.61	Phe 1TMS	20.25	Mal 3TMS	27.48	Pyr 1M 1TMS	42.53
Ace 2TMS	44.20	Pyr 1M 1TMS	26.26	Pyr 1M 1TMS	34.19	Ace 2TMS	111.82
Hip 2TMS	N/D	Hip 2TMS	N/D	Hip 2TMS	N/D	Hip 2TMS	N/D

Starting with the adapted thermal derivatisation condition evaluating 50°C for the reaction time of 30 min, 17 derivatives displayed a CV ≤ 20%; for 60 min, 21 derivatives; for 90 min, 20 derivatives and for 120 min, 20 derivatives resulted in a CV value ≤ 20% respectively (Table 4.5). In Table 4.6 the temperature investigating 60°C is given. A reaction time of 30 min resulted in 20 derivatives with a CV ≤ 20%; for 60 min, 19 derivatives; for 90 min, 19 derivatives and for 120 min, 19 derivatives had a CV value ≤ 20% respectively. In Table 4.7 at the derivatisation temperature of 70°C, a reaction time of 30 min resulted in 18 derivatives with a CV ≤ 20%; for 60 min, 18 derivatives; for 90 min, 19 derivatives and for 120 min, 18 derivatives had a CV value ≤ 20% respectively. Lastly, the temperature investigating 85°C with a reaction time of 30 min showed 20 derivatives with a CV ≤ 20%; for 60 min, 21 derivatives; for 90 min, 19 derivatives and for 120 min, 20 derivatives had a CV value ≤ 20% respectively (Table 4.8).

Both the lowest (50°C) and highest (85°C) temperatures investigated at a reaction time of 60 min, resulted in the largest number of derivatives with a CV ≤ 20%. At 60°C and 70°C, the reaction times of 30 min and 90 min resulted in the largest number of derivatives with a CV ≤ 20% respectively. Overall the number of derivatives with a CV ≤ 20% are almost the same for all investigated temperatures, between reaction times. The condition with the lowest number of derivatives with a CV ≤ 20% is 50°C at 30 min, which is the lowest temperature with the shortest reaction time investigated. However, some compounds are represented by multiple derivatives, which is not

recommended outcome (as discussed in Section 4.2.4.2) when striving for repeatability derivatisation (Bekele *et al.*, 2014). It was further observed that some of the multiple derivatives for a compound (N-acetyl-L-alanine, citric acid, hippuric acid, 2-ketoglutaric acid, malonic acid and succinylacetone) resulted in low signal-to-noise levels with low relative response values leading to high CV values in the data. More focus on the derivatisation outcome of specific compounds will be discussed later (Section 4.2.5).

Notably, with the addition of methoxyamine, the internal standard pentadecanoic acid, displayed a CV ≤ 20% for all adapted thermal conditions. Pentadecanoic acid shows good repeatability due to the fact that this compound does not present different derivatives following derivatisation (Kouremenos *et al.*, 2010a). However, the addition of sample extraction to the experimental procedure might result in a different outcome. Palmitic acid 1TMS displayed lower CV < 3% for all conditions, although good repeatability was achieved for this compound it is not typically used as an internal standard since it is a metabolite that is present in biological fluids (Bouatra *et al.*, 2013). The CV values for the most commonly used internal standard, 3-phenylbutyric acid, ranged from 3.42 - 26.16% (Tables 4.5 - 4.8) as discussed in Section 4.2.5.

Dicarboxylic acids without added functional groups in Tables 4.5 - 4.8 show that adipic acid 2TMS, fumaric acid 2TMS and sebacic acid 2TMS have a CV < 6% at all conditions investigated. For adipic acid and sebacic acid derivatisation including the use of oxymation (room temperature for 60 min) and silylation (80°C for 30 min) were compared with and without applying flash heater derivatisation (Nakagawa *et al.*, 2010). Nakagawa *et al.* (2010) reported CV values of 4.7% and 4.0% for adipic acid and sebacic acid with the use of flash heater derivatisation and without its use, CV values of 2.7% for adipic acid and 2.0% for sebacic acid were reported. These low CV values makes the use of oxymation an attractive alternative when derivatising these two compounds, however similar CV values were achieved in the current study using methoxyamine. Furthermore, the dicarboxylic acid oxalic acid 2TMS shows a CV that ranges from 8.00 to 30.44% (Tables 4.5 - 4.8), and cis-aconitic acid 3TMS provided a CV < 10% at all adapted thermal conditions (Tables 4.5 - 4.8).

Although orotic acid consists of two keto- groups, these groups were not methoxymated. Instead a tri- TMS derivative was formed due to silylation of the carboxyl and or keto- or the amino groups. CV values were found to be ≤ 20% for all investigated conditions except for the 50°C for 30 min combination for which the CV was 33.60%. The CV value > 20% obtained for orotic acid at the lowest temperature and shortest reaction time (50°C for 30 min) shows that this compound performs better at the higher temperatures and longer derivatisation times. The keto- group of pyruvic acid caused methoxymation at this site (Lai and Fiehn, 2016) which can explain the result of pyruvic acid

derivative with only one TMS group. For pyruvic acid 1M, 1TMS all conditions displayed CV > 20% with values ranging from 21.49 to 84.32%.

The use of methoxymation is recommended by Fiehn *et al.* (2000) and Kaluzna-Czaplińska (2011) to prevent the formation of multiple derivatives of keto- acids. Tables 4.5 - 4.8. show that multiple derivatives i.e. N-acetyl-L-alanine, citric acid, hippuric acid, 2-ketoglutaric acid, malonic acid and succinylacetone still resulted in more than one derivative from the same compound following the use of methoxyamine. Important to consider is that citric acid and malonic acid do not consist of a keto- group (Table 2.3), but also formed multiple derivatives. The CV for N-acetyl-L-alanine 1TMS is < 5% for all conditions, while N-acetyl-L-alanine 2TMS had a CV > 20% for all conditions excluding 85°C for 60 min as an example of the results obtained. Overall the results show that the derivatisation with the addition of methoxyamine caused less variation for N-acetyl-L-alanine 1TMS than for the 2TMS derivative. Citric acid formed two derivatives, with citric acid 3TMS resulting in CV values ≤ 20% for seven of the conditions investigated. The citric acid 4TMS derivative shows CV ≤ 20% for all conditions except at 70°C for 60 min (Tables 4.5 - 4.8) were the CV values resulted in 20.06%. Better repeatability was obtained for citric acid 4TMS when using the adapted thermal derivatisation conditions.

As in Section 4.2.1 hippuric acid subjected to adapted thermal derivatisation resulted in the formation of two derivatives at certain conditions. The mono- TMS derivative has a CV ≤ 20% for all conditions with the exception seen at 50°C for 120 min (33.53%) and the di- TMS derivative also showed a CV value exeeding 20% at all conditions. Interestingly, Tables 4.5 - 4.8 show that hippuric acid 2TMS was only detected at 60°C, but with high CV values ranging from 39.90 - 93.99% indicating instability. Gerlo *et al.*, (2006) reported that poorly shaped peaks were obtained for nitrogen containing compounds, such as hippuric acid, that suggests instability of the TMS derivatives. Often when small peaks with low signal-to-noise ratios are integrated, low relative responses result in a high CV values (Amigo *et al.*, 2008), as was seen in this study. Within all investigated conditions, the mono- TMS derivative for hippuric acid provided the best repeatability (Tables 4.5 - 4.8).

The compound 2-ketoglutaric acid resulted in two derivatives for all conditions investigated (Tables 4.5 - 4.8), with both isoforms (1M 2TMS) resulting in CV ≤ 20%. It was found that methoxymation of the keto- group prevents cyclisation (Fiehn *et al.*, 2000), and keto- enol tautomerisation occurs (Lai and Fiehn, 2016). However, as syn and anti isoforms can be found for 2-ketoglutaric acid, (Qiu and Reed, 2014) two derivatives were still the outcome following adapted thermal derivatisation.

In Tables 4.5 - 4.8 the difference in derivatisation efficiency of malonic acid 2TMS and malonic acid 3TMS under adapted thermal derivatisation are displayed. All CV values for the di- TMS derivative

of malonic acid are ≤ 20%. The CV values for the tri- TMS derivative of malonic acid ranged from 6.71 to 27.48%, with seven conditions > 20%. For three conditions the tri- TMS derivative provided lower CV values than the di- TMS derivative. These results indicate that temperature nor reaction time influenced the derivatisation efficiency between the two derivatives of malonic acid.

Succinylacetone is an example of a compound which provided CV ≤ 20% for all four isomer derivatives detected within all of the adapted thermal conditions investigated. Syn and anti isoforms (succinylacetone 1M 1TMS a,b,c or d) seem probable considering the chemical structure of succinylacetone where both of the keto- groups could be methoxymated (Qiu and Reed, 2014).

4.2.3 Microwave-assisted derivatisation

As described in Section 3.4.3 the microwave-assisted derivatisation experiments were performed at four pre-selected microwave energies (150, 230, 350 and 450 W), each at four different reaction times (1.5, 2.0, 3.0 and 4.0 min) (Table 3.4). Each condition also consisted of a batch with five replicates.

4.2.3.1 Determination of outliers

The relative response data obtained from microwave-assisted derivatisation was evaluated for the presence of outliers within the analysed replicates for all conditions (as described in Section 4.2.1.1). The PCA scores plot (A) and Hotelling's T^2 distance plot (B) of five replicates for the microwave-assisted derivatisation at the condition of 150 W for 1.5 min are shown in Figure 4.6. Plots representing the other microwave-assisted derivatisation conditions investigated are given in Appendix A (Figs. A.31 - A.45).

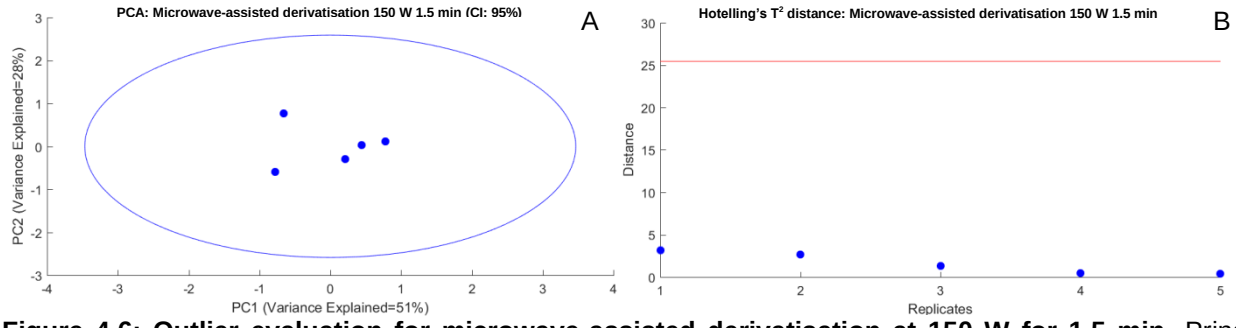


Figure 4.6: Outlier evaluation for microwave-assisted derivatisation at 150 W for 1.5 min. Principal component analysis (A) and Hotelling's T^2 (B) for five replicates in a batch.

Following the preparation and analysis of the same sample in five replicates the PCA scores plot in Figure 4.6A accounts for 79% of the total variance in the data. Together with the Hotelling's T^2 distance plot (Fig. 4.6B) the data confirms that the samples are compositionally similar to each other. Considering that all of the data are grouped within the confidence interval and confidence threshold

it is possible to say that no outliers were present for this microwave-assisted derivatisation condition (similar to the findings of Section 4.2.1.1). Furthermore, the other microwave-assisted derivatisation conditions investigated (Appendix A, Figs. A.31 - A.45) followed the same pattern, confirming that no outliers were present in all of the replicates analysed, ensuring the inclusion of all collected data for further data analysis.

4.2.3.2 Comparison of within condition repeatability

As in Section 4.2.1.2, CV calculations were used to establish the repeatability within each microwave-assisted condition (specific temperature and reaction time combination), which resulted in the overview of calculated CV values (%) in Figure 4.7.

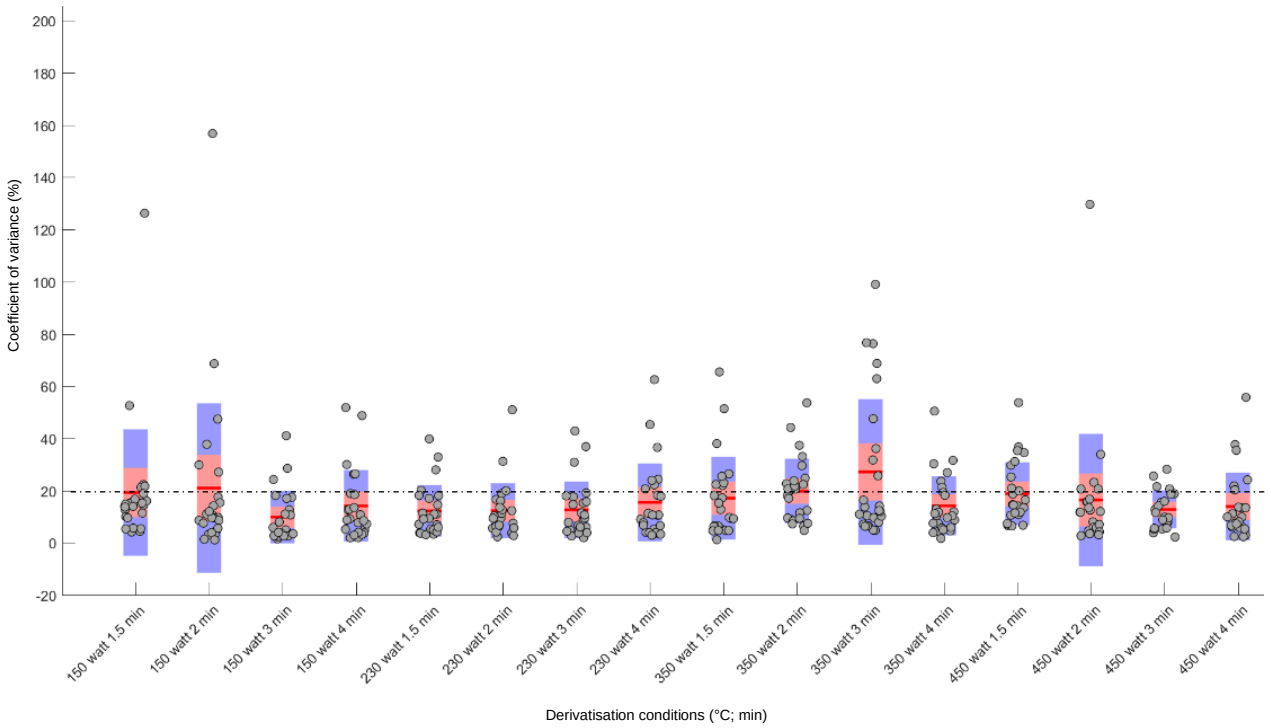


Figure 4.7: Box-like plots of conditions evaluated for microwave-assisted derivatisation. Microwave-assisted derivatisation conditions are shown on the x-axis. At each increment the first value indicate microwave energy (Watt) and second value indicate reaction time (min). Coefficient of variance (%) is shown on the y-axis. The black dotted line indicates the CV ≤ 20% cut-off.

As presented in the box-like-plots in Figure 4.7, it is clear that none of the investigated conditions resulted in repeatable CV values ≤ 20% for all of the 25 derivatives representing the 16 selected compounds. Based on the evidence, 13 of the 16 conditions show an average CV ≤ 20%. The three conditions with average CV > 20%, include 150 W, 2.0 min; 350 W, 1.5 min and 350 W, 3.0 min. For compounds with more than one derivative, the CV may vary since derivatisation efficiency is dependent on microwave energy and reaction time (Kouremenos *et al.*, 2010a). To exclude the possibility of skewed data, each derivative will be discussed separately.

Tables 4.9 - 4.12 show the calculated CV values for each compound according to its associated derivative(s), in order of the different derivatisation microwave energies, i.e. 150, 230, 350 and 450 W. The derivatives are listed based on increasing CV values and a 20% CV cut-off value is indicated by the bold line within the columns.

Table 4.9: Coefficient of variance for microwave-assisted derivatives at 150 W

1.5 min	CV (%)	2.0 min	CV (%)	3.0 min	CV (%)	4.0 min	CV (%)
Fum 2TMS	4.15	Pal 1TMS	1.11	Ket 1TMS	1.49	Fum 2TMS	1.89
Ace 1TMS	4.37	Pen 1TMS	1.36	Ace 1TMS	2.38	Ace 1TMS	1.94
Seb 2TMS	5.30	Fum 2TMS	3.25	Phe 1TMS	2.43	Seb 2TMS	2.94
Adi 2TMS	5.40	Adi 2TMS	3.36	Ket 3TMS	2.55	Adi 2TMS	3.22
Aco 3TMS	5.61	Seb 2TMS	3.85	Aco 3TMS	2.60	Suc 2TMS c	3.75
Pen 1TMS	9.55	Hip 2TMS	4.57	Seb 2TMS	2.66	Aco 3TMS	3.98
Ket 3TMS	10.00	Aco 3TMS	5.56	Fum 2TMS	3.38	Suc 2TMS a	4.92
Hip 2TMS	11.38	Ket 2TMS	7.52	Suc 2TMS c	3.43	Suc 2TMS b	5.15
Suc 2TMS c	12.70	Mal 2TMS	8.58	Pen 1TMS	3.45	Pal 1TMS	7.20
Phe 1TMS	13.70	Suc 2TMS a	8.61	Adi 2TMS	3.64	Cit 3TMS	7.65
Suc 2TMS a	13.89	Ket 3TMS	9.55	Suc 2TMS a	3.98	Pen 1TMS	8.48
Pal 1TMS	13.92	Cit 3TMS	9.63	Pal 1TMS	4.00	Mal 2TMS	8.73
Ket 1TMS	14.44	Suc 2TMS c	9.65	Suc 2TMS b	4.93	Ket 3TMS	9.17
Mal 2TMS	14.79	Suc 2TMS b	10.28	Cit 3TMS	5.86	Hip 2TMS	10.72
Ket 2TMS	15.08	Suc 3TMS	11.00	Ket 2TMS	7.97	Ket 1TMS	12.59
Cit 4TMS	15.23	Phe 1TMS	12.01	Mal 2TMS	10.65	Ace 2TMS	12.83
Suc 2TMS b	15.93	Cit 4TMS	14.14	Hip 2TMS	10.99	Phe 1TMS	13.46
Cit 3TMS	16.05	Ace 1TMS	15.35	Suc 3TMS	12.65	Ket 2TMS	18.43
Oro 3TMS	16.69	Ket 1TMS	17.56	Ace 2TMS	16.97	Suc 3TMS	18.85
Oxa 2TMS	18.84	Mal 3TMS	27.07	Mal 3TMS	17.43	Mal 3TMS	26.23
Mal 3TMS	21.24	Oro 3TMS	29.83	Cit 4TMS	18.10	Cit 4TMS	26.39
Ace 2TMS	21.67	Ace 2TMS	37.71	Oro 3TMS	24.22	Oxa 2TMS	29.96
Suc 3TMS	22.26	Pyr 2TMS	47.39	Pyr 2TMS	28.51	Pyr 2TMS	48.73
Pyr 2TMS	52.56	Oxa 2TMS	68.62	Oxa 2TMS	41.00	Oro 3TMS	51.80
Hip 1TMS	126.24	Hip 1TMS	156.77	Hip 1TMS	N/D	Hip 1TMS	N/D

Table 4.10: Coefficient of variance for microwave-assisted derivatives at 230 W

1.5 min	CV (%)	2.0 min	CV (%)	3.0 min	CV (%)	4.0 min	CV (%)
Adi 2TMS	3.09	Fum 2TMS	2.27	Adi 2TMS	1.92	Pal 1TMS	2.90
Ace 1TMS	3.26	Adi 2TMS	2.73	Fum 2TMS	2.58	Pen 1TMS	3.27
Fum 2TMS	3.63	Ace 1TMS	3.73	Pal 1TMS	3.71	Fum 2TMS	3.64
Suc 2TMS a	3.78	Seb 2TMS	4.10	Seb 2TMS	3.87	Seb 2TMS	3.84
Pal 1TMS	4.14	Mal 2TMS	5.58	Ace 1TMS	4.02	Adi 2TMS	3.85
Seb 2TMS	4.95	Pal 1TMS	5.68	Phe 1TMS	4.39	Ace 1TMS	3.97
Oxa 2TMS	5.29	Cit 4TMS	6.21	Suc 2TMS a	4.72	Ket 3TMS	5.23
Suc 2TMS b	5.30	Suc 3TMS	6.21	Suc 2TMS b	5.96	Aco 3TMS	6.53
Pen 1TMS	5.93	Pen 1TMS	6.71	Pen 1TMS	6.01	Phe 1TMS	6.74
Cit 4TMS	6.88	Ket 1TMS	7.46	Aco 3TMS	6.70	Hip 2TMS	7.67
Ket 1TMS	7.08	Aco 3TMS	9.38	Mal 2TMS	7.80	Suc 2TMS c	9.01
Aco 3TMS	9.05	Suc 2TMS b	9.48	Suc 2TMS c	8.87	Suc 2TMS a	10.54
Cit 3TMS	9.31	Hip 1TMS	10.25	Ket 1TMS	9.47	Oro 3TMS	10.65
Hip 1TMS	10.96	Suc 2TMS c	11.27	Ket 3TMS	10.67	Mal 2TMS	10.65
Ket 3TMS	10.98	Suc 2TMS a	11.64	Ket 2TMS	11.46	Suc 2TMS b	11.32
Mal 2TMS	11.35	Oxa 2TMS	12.30	Oxa 2TMS	14.63	Ace 2TMS	17.79
Ket 2TMS	14.54	Ket 3TMS	12.64	Mal 3TMS	15.25	Ket 1TMS	18.20
Suc 3TMS	16.99	Cit 3TMS	13.54	Cit 3TMS	15.72	Cit 3TMS	20.64
Mal 3TMS	18.08	Ace 2TMS	16.07	Pyr 2TMS	17.57	Ket 2TMS	22.17
Phe 1TMS	18.15	Mal 3TMS	16.58	Cit 4TMS	17.96	Mal 3TMS	23.90
Suc 2TMS c	20.06	Ket 2TMS	18.89	Hip 1TMS	19.07	Suc 3TMS	24.27
Pyr 2TMS	27.92	Phe 1TMS	19.95	Oro 3TMS	30.85	Cit 4TMS	36.52
Ace 2TMS	32.83	Pyr 2TMS	31.18	Suc 3TMS	36.83	Oxa 2TMS	45.29
Oro 3TMS	39.79	Oro 3TMS	50.95	Ace 2TMS	42.83	Pyr 2TMS	62.47
Hip 2TMS	N/D	Hip 2TMS	N/D	Hip 2TMS	N/D	Hip 1TMS	N/D

Table 4.11: Coefficient of variance for all microwave-assisted derivatives at 350 W

1.5 min	CV (%)	2.0 min	CV (%)	3.0 min	CV (%)	4.0 min	CV (%)
Adi 2TMS	1.17	Aco 3TMS	4.71	Ace 1TMS	4.74	Oro 3TMS	1.67
Fum 2TMS	4.57	Fum 2TMS	6.38	Adi 2TMS	4.88	Adi 2TMS	3.86
Aco 3TMS	4.69	Seb 2TMS	7.22	Seb 2TMS	6.29	Ace 1TMS	4.36
Suc 2TMS c	4.71	Pen 1TMS	7.45	Oro 3TMS	6.38	Ket 3TMS	4.54
Seb 2TMS	4.73	Adi 2TMS	8.36	Fum 2TMS	6.43	Seb 2TMS	4.98
Suc 2TMS a	5.66	Ket 3TMS	8.88	Aco 3TMS	7.10	Aco 3TMS	5.68
Ace 1TMS	6.20	Pal 1TMS	9.35	Hip 2TMS	7.89	Fum 2TMS	5.86
Suc 2TMS b	6.38	Hip 2TMS	9.48	Ace 2TMS	9.62	Mal 2TMS	7.42
Ket 3TMS	6.48	Oxa 2TMS	11.47	Phe 1TMS	10.18	Mal 3TMS	7.47
Pen 1TMS	9.27	Ace 2TMS	12.39	Suc 2TMS a	10.40	Suc 2TMS a	8.74
Phe 1TMS	9.38	Ket 2TMS	17.03	Suc 2TMS c	10.91	Ket 1TMS	9.61
Pal 1TMS	9.68	Suc 3TMS	18.06	Pal 1TMS	11.99	Suc 2TMS c	10.30
Ket 1TMS	12.72	Suc 2TMS a	19.27	Pen 1TMS	12.35	Suc 3TMS	10.40
Hip 2TMS	15.20	Suc 2TMS c	20.55	Suc 2TMS b	13.71	Phe 1TMS	11.18
Ace 2TMS	17.19	Ket 1TMS	20.62	Suc 3TMS	14.06	Ace 2TMS	11.69
Ket 2TMS	18.03	Mal 3TMS	21.70	Mal 2TMS	16.35	Suc 2TMS b	11.88
Mal 2TMS	21.93	Phe 1TMS	22.32	Mal 3TMS	25.65	Ket 2TMS	12.86
Mal 3TMS	22.32	Suc 2TMS b	22.65	Hip 1TMS	31.70	Pen 1TMS	18.20
Cit 4TMS	22.92	Cit 4TMS	23.74	Ket 3TMS	36.08	Pal 1TMS	19.24
Oro 3TMS	25.43	Cit 3TMS	24.74	Ket 2TMS	47.53	Oxa 2TMS	21.22
Suc 3TMS	26.41	Ace 1TMS	29.55	Ket 1TMS	62.86	Hip 2TMS	23.54
Cit 3TMS	38.01	Hip 1TMS	33.03	Cit 3TMS	68.70	Cit 4TMS	26.89
Oxa 2TMS	51.37	Mal 2TMS	37.33	Pyr 2TMS	76.27	Cit 3TMS	30.25
Pyr 2TMS	65.44	Pyr 2TMS	44.16	Oxa 2TMS	76.65	Hip 1TMS	31.57
Hip 1TMS	N/D	Oro 3TMS	53.59	Cit 4TMS	99.02	Pyr 2TMS	50.43

Table 4.12: Coefficient of variance for microwave-assisted derivatives at 450 W

1.5 min	CV (%)	2.0 min	CV (%)	3.0 min	CV (%)	4.0 min	CV (%)
Aco 3TMS	6.42	Adi 2TMS	2.69	Adi 2TMS	2.20	Adi 2TMS	2.32
Ace 1TMS	6.56	Pen 1TMS	3.04	Fum 2TMS	3.79	Fum 2TMS	2.40
Fum 2TMS	6.70	Seb 2TMS	3.48	Aco 3TMS	5.16	Ace 1TMS	2.93
Adi 2TMS	6.97	Pal 1TMS	4.17	Ace 1TMS	5.22	Seb 2TMS	5.32
Seb 2TMS	7.33	Ket 3TMS	4.60	Pal 1TMS	5.56	Ket 1TMS	5.79
Ket 3TMS	10.55	Cit 4TMS	5.04	Seb 2TMS	5.65	Suc 2TMS b	5.96
Ket 2TMS	10.75	Ket 1TMS	5.64	Suc 2TMS a	7.87	Ket 3TMS	6.02
Pen 1TMS	11.42	Fum 2TMS	5.82	Ket 3TMS	8.14	Mal 2TMS	6.63
Suc 2TMS a	11.57	Ace 2TMS	5.95	Pen 1TMS	8.77	Suc 2TMS a	6.84
Pal 1TMS	11.73	Aco 3TMS	7.01	Cit 4TMS	9.60	Pen 1TMS	7.22
Suc 2TMS c	13.48	Ace 1TMS	8.01	Suc 2TMS b	9.84	Suc 2TMS c	7.49
Suc 2TMS b	14.07	Pyr 2TMS	11.70	Oxa 2TMS	11.55	Pal 1TMS	8.10
Phe 1TMS	14.45	Ket 2TMS	11.73	Suc 2TMS c	12.74	Ket 2TMS	9.86
Cit 4TMS	14.62	Suc 2TMS c	11.98	Ket 2TMS	14.09	Aco 3TMS	9.90
Ace 2TMS	16.45	Suc 3TMS	12.52	Suc 3TMS	15.54	Mal 3TMS	11.11
Suc 3TMS	18.63	Phe 1TMS	13.46	Phe 1TMS	15.87	Hip 1TMS	13.33
Ket 1TMS	19.83	Suc 2TMS a	15.75	Cit 3TMS	18.62	Oxa 2TMS	13.42
Hip 2TMS	20.90	Suc 2TMS b	15.82	Mal 3TMS	18.79	Cit 3TMS	13.64
Mal 2TMS	25.20	Mal 3TMS	16.72	Ket 1TMS	19.00	Phe 1TMS	20.27
Hip 1TMS	29.72	Cit 3TMS	20.58	Pyr 2TMS	20.63	Suc 3TMS	21.74
Cit 3TMS	31.15	Oxa 2TMS	20.59	Mal 2TMS	21.50	Cit 4TMS	24.09
Oro 3TMS	34.54	Mal 2TMS	23.16	Oro 3TMS	25.55	Ace 2TMS	35.42
Oxa 2TMS	35.30	Oro 3TMS	33.85	Hip 1TMS	28.15	Pyr 2TMS	37.60
Mal 3TMS	36.76	Hip 1TMS	45.21	Ace 2TMS	N/D	Oro 3TMS	55.69
Pyr 2TMS	53.72	Hip 2TMS	N/D	Hip 2TMS	N/D	Hip 2TMS	N/D

In Table 4.9 the microwave energy conducted at 150 W is illustrated where a reaction time of 1.5 min resulted in 20 derivatives with a CV ≤ 20%; for 2.0 min, 19 derivatives; for 3.0 min, 21 derivatives and for 4.0 min, 19 derivatives had a CV value ≤ 20% respectively. Table 4.10 displays the microwave energy investigating 230 W, with a reaction time of 1.5 min resulting in 20 derivatives with a CV ≤ 20%; for 2.0 min, 22 derivatives; for 3.0 min, 21 derivatives and for 4.0 min, 17 derivatives had a CV value ≤ 20% respectively. In Table 4.11 at a derivatisation microwave energy of 350 W, a reaction time of 1.5 min resulted in 16 derivatives with a CV ≤ 20%; for 2.0 min, 13 derivatives; for 3.0 min, 16 derivatives and for 4.0 min, 19 derivatives had a CV value ≤ 20%. Lastly, Table 4.12 reflects the microwave energy at 450 W, using a reaction time of 1.5 min, where 17 derivatives displayed a CV ≤ 20%; for 2.0 min, 19 derivatives; for 3.0 min, 19 derivatives and for 4.0 min, 18 derivatives had respective CV values ≤ 20%.

The largest number of derivatives with a CV ≤ 20% at 150 W were found after 3.0 min of derivatisation. Using CV ≤ 20% as measurement, derivatisation at 230 W after 2.0 min, 350 W after 4.0 min and 450 W at both 2.0 and 3.0 min resulted in the largest number of derivatives, indicating the most repeatable conditions. From this it is clear that the number of derivatives with a CV ≤ 20% greatly varies between the investigated microwave energies and reaction times as noted previously derivatisation of organic acids result in different derivatisation efficiency at different conditions

(Kouremenos *et al.*, 2010a). As a means to elucidate this, the discussion will continue by focusing on individual derivatives as seen in Tables 4.9 - 4.12.

The derivative for pentadecanoic acid displayed CV ≤ 20% indicating good repeatability for all conditions. The condition at 350 W for 4.0 min display the highest CV value (18.20%) but does not show an increase or decrease towards certain microwave energies nor reaction times. Other monocarboxylic acids showed a CV ≤ 20% for palmitic acid and CV < 23% for 3-phenylbutyric acid at all conditions investigated (Tables 4.9 - 4.12).

When focusing on the, dicarboxylic acids with no additional functional groups (Table 2.3), adipic acid 2TMS, fumaric acid 2TMS and sebacic acid 2TMS have a CV < 10% at all conditions investigated (Tables 4.9 - 4.12). Then again oxalic acid 2TMS shows CV values that range from 5.29 to 76.65% and cis-aconitic acid 3TMS provided repeatable derivatisation with a CV < 10% at all microwave-assisted conditions investigated (Tables 4.9 - 4.12). Orotic acid formed a tri- TMS derivative when subjected to microwave-assisted derivatisation, with the highest CV (55.69%) obtained at 450 W for 4.0 min and the lowest CV (1.67%) at 350 W for 4.0 min. For the di- TMS derivative of pyruvic acid, the CV values were ≤ 20% obtained at two conditions (at 450 W for 2.0 min and at 230 W for 3.0 min) and the pyruvic acid 2TMS derivative greatly varies with CV values ranging from 11.70 to 76.27%. This is unlike the findings by Jurado-Sánchez *et al.* (2012) where the analysis of samples resulted in a within-in day standard deviation of 4% for pyruvic acid using BSTFA-TMCS (99:1%).

Again, from Tables 4.9 - 4.12 it is evident that N-acetyl-L-alanine, citric acid, hippuric acid, 2-ketoglutaric acid, malonic acid and succinylacetone resulted in more than one derivative for the same compound following microwave-assisted derivatisation. N-acetyl-L-alanine 1TMS showed a CV > 20% for one condition (350 W for 2.0 min). For N-acetyl-L-alanine 2TMS five conditions displayed CV > 20% and in one condition (450 W for 3.0 min) the derivative was not detected. Overall the results show that N-acetyl-L-alanine 1TMS causes less variation than the di- TMS derivative. Citric acid is another organic acid for which two derivatives were formed. The tri- TMS form of citric acid showed CV values ranging from 5.86 to 68.70% and for citric acid 4TMS the CV ranged from 5.04 to 99.02%. The derivative hippuric acid 1TMS showed a CV ≤ 20% for only four conditions and was not detected at four conditions. Hippuric acid 2TMS displayed a CV ≤ 20% for eight conditions and was not detected at six conditions. For both derivatives the remaining conditions resulted in CV values > 20%.The instability of hippuric acid experienced in this study can be due to the fact that nitrogen consisting compounds are often associated with unstable derivatives (Gerlo *et al.*, 2006).

In Tables 4.9 - 4.12 CV values for 2-ketoglutaric acid 1TMS vary from 1.49 to 62.86%, for 2-ketoglutaric acid 2TMS CV values range from 7.52.42 to 47.53% and for 2-ketoglutaric acid 3TMS

CV values range from 2.55 to 36.08%. Most conditions indicate less variation for 2-ketoglutaric acid 3TMS than the other two derivatives. The CV values for the di- TMS derivative of malonic acid range from 5.58 to 37.33%, with five conditions that have CV values above 20%. The CV values for the tri- TMS derivative of malonic acid range from 7.47 to 36.76%, with eight conditions above 20%. For three conditions the tri- TMS derivative provided lower CV values than for the di- TMS derivative. The CV for succinylacetone 3TMS vary between 6.21 to 36.83%, with a CV above 20% for five of the tested conditions. The investigated conditions failed to eliminate formation of multiple derivatives for succinylacetone following microwave-assisted derivatisation.

4.2.4 Adapted microwave-assisted derivatisation

As described in Section 3.4.4 the adapted microwave-assisted derivatisation experiments were performed at four pre-selected microwave energies (150, 230, 350 and 450 W), at four different reaction times (1.5, 2.0, 3.0 and 4.0 min) (Table 3.5), consisting of five replicates in each batch.

4.2.4.1 Determination of outliers

The presence of outliers within all analysed replicates and conditions (Section 4.2.1.1) were evaluated using the relative response data resulting from adapted microwave-assisted derivatisation. Using the first adapted microwave-assisted condition (150 W; 1.5 min) investigated Figure 4.8 illustrates the data of five replicates as PCA scores plot (A) and Hotelling's T^2 distance plot (B), with other conditions illustrated in Appendix A (Figs. A.46 - A.60).

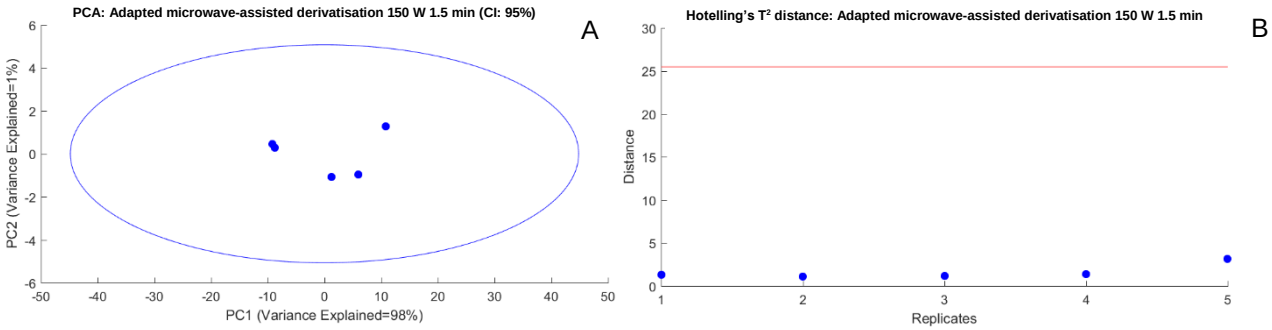


Figure 4.8: Outlier evaluation for adapted microwave-assisted derivatisation at 150 W for 1.5 min. Principal component analysis (A) and Hotelling's T^2 (B) for five replicates in a batch.

In Figure 4.8A, the PCA scores plot explains 99% of the total variance in the data, and the Hotelling's T^2 distance plot (Fig. 4.8B) supports the absence of outliers in this adapted microwave-assisted derivatisation dataset (as in Section 4.2.1). Again, the other adapted microwave-assisted investigated conditions in Appendix A, Figures A.46 - A.60, show samples with similar composition without outliers, enabling the use thereof for remaining data analysis.

4.2.4.2 Comparison of within condition repeatability

To compare the repeatability within each adapted microwave-assisted condition (specific temperature and reaction time combination), CV was calculated as described in Section 4.2.1.2. Figure 4.9 shows the calculated CV (%) values for all conditions investigated within the adapted microwave-assisted derivatisation experiment.

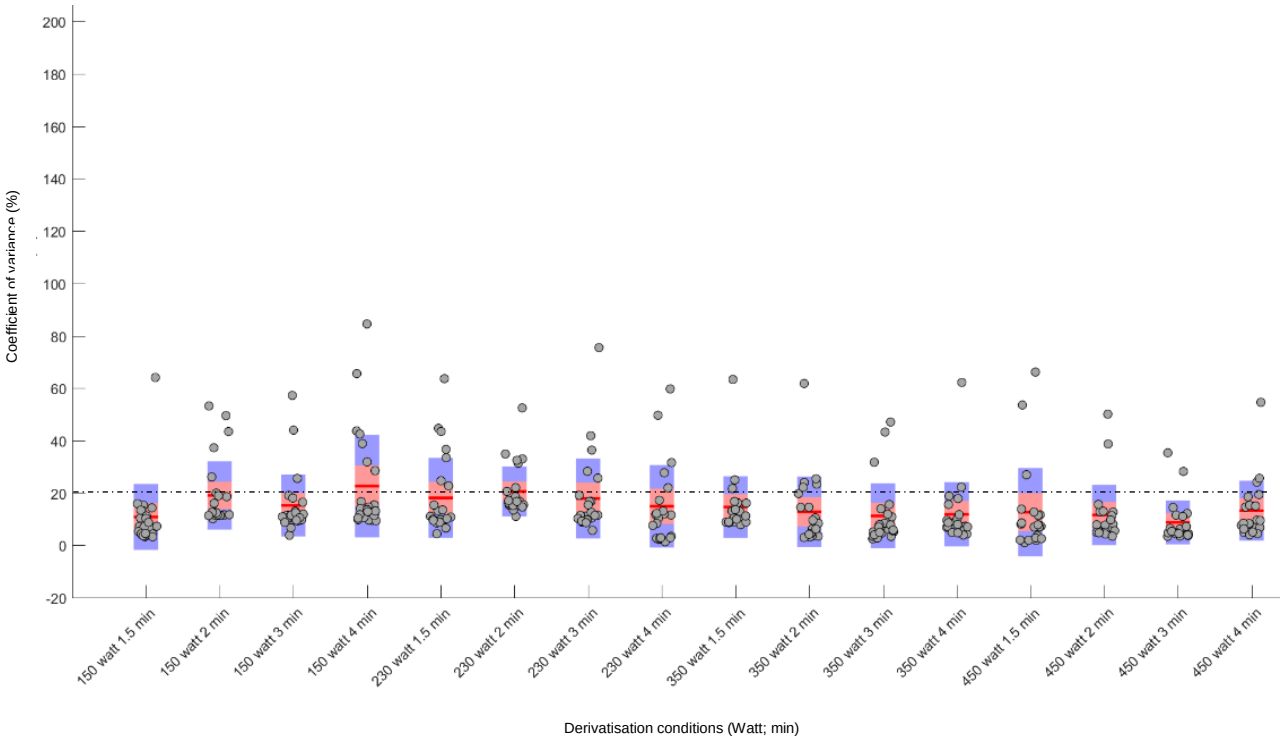


Figure 4.9: Box-like plots of conditions evaluated for adapted microwave-assisted derivatisation. Adapted microwave-assisted derivatisation conditions are shown on the x-axis. At each increment the first value indicate microwave energy (Watt) and second value indicate reaction time (min). Coefficient of variance (%) is shown on the y-axis. The black dotted line indicates the CV ≤ 20% cut-off.

The box-like plots in Figure 4.9, show that repeatable CV values ≤ 20% for all 24 derivatives (from the 16 preselected compounds) were not achieved by any of the tested conditions. A total 14 conditions resulted in an average CV ≤ 20%. The two conditions with average CV > 20%, were 150 W for 4.0 min and 230 W for 2.0 min. Considering that the data may be skewed due to multiple derivatives from the same compounds, derivatives will be focused on individually in the next sections.

Tables 4.13 - 4.16 display the CV values calculated for each compound (and associated derivatives) according to different derivatisation microwave energies 150, 230, 350 and 450 W. Increasing CV values were used to order derivatives detected, and a 20% CV cut-off value was used to distinguish repeatability.

Table 4.13: Coefficient of variance for adapted microwave-assisted derivatives at 150 W

1.5 min	CV (%)	2.0 min	CV (%)	3.0 min	CV (%)	4.0 min	CV (%)
Fum 2TMS	3.26	Phe 1TMS	10.19	Mal 2TMS	3.83	Phe 1TMS	9.38
Ace 1TMS	3.29	Hip 1TMS	10.99	Cit 4TMS	6.70	Suc 1M 1TMS b	9.50
Pal 1TMS	3.69	Suc 1M 1TMS c	11.13	Pen 1TMS	8.73	Suc 1M 1TMS d	9.63
Seb 2TMS	3.74	Suc 1M 1TMS b	11.30	Ket 1M 2TMS b	9.30	Suc 1M 1TMS c	9.78
Cit 4TMS	4.31	Pen 1TMS	11.37	Pal 1TMS	9.56	Pen 1TMS	10.40
Aco 3TMS	4.34	Adi 2TMS	11.48	Adi 2TMS	9.80	Ace 1TMS	10.65
Pen 1TMS	4.55	Oro 3TMS	11.60	Ace 1TMS	10.40	Aco 3TMS	11.35
Ket 1M 2TMS b	5.27	Ace 1TMS	11.60	Seb 2TMS	10.53	Fum 2TMS	11.70
Suc 1M 1TMS a	6.94	Cit 3TMS	11.61	Fum 2TMS	10.84	Suc 1M 1TMS a	11.99
Suc 1M 1TMS b	6.98	Suc 1M 1TMS d	11.63	Aco 3TMS	10.90	Adi 2TMS	12.67
Suc 1M 1TMS d	7.19	Fum 2TMS	11.67	Ket 1M 2TMS a	11.13	Pal 1TMS	12.88
Suc 1M 1TMS c	7.26	Aco 3TMS	11.68	Suc 1M 1TMS d	11.33	Seb 2TMS	13.07
Adi 2TMS	8.74	Seb 2TMS	12.42	Suc 1M 1TMS b	11.36	Oro 3TMS	13.65
Cit 3TMS	10.27	Pal 1TMS	12.59	Phe 1TMS	11.49	Ket 1M 2TMS a	14.09
Ket 1M 2TMS a	10.41	Suc 1M 1TMS a	12.71	Suc 1M 1TMS c	11.55	Cit 4TMS	14.61
Hip 1TMS	11.18	Ket 1M 2TMS a	16.06	Oro 3TMS	11.90	Mal 2TMS	15.44
Mal 2TMS	13.29	Cit 4TMS	18.57	Oxa 2TMS	12.50	Ket 1M 2TMS b	16.69
Oxa 2TMS	13.42	Mal 2TMS	18.93	Suc 1M 1TMS a	13.07	Cit 3TMS	28.51
Oro 3TMS	14.30	Ket 1M 2TMS b	19.91	Hip 1TMS	16.44	Hip 1TMS	31.87
Pyr 1M 1TMS	15.25	Ace 2TMS	26.11	Cit 3TMS	18.03	Oxa 2TMS	38.86
Phe 1TMS	15.90	Oxa 2TMS	37.23	Hip 2TMS	19.07	Ace 2TMS	42.49
Mal 3TMS	64.08	Mal 3TMS	43.43	Ace 2TMS	25.56	Mal 3TMS	43.68
Ace 2TMS	N/D	Hip 2TMS	49.51	Pyr 1M 1TMS	44.00	Pyr 1M 1TMS	65.57
Hip 2TMS	N/D	Pyr 1M 1TMS	53.20	Mal 3TMS	57.24	Hip 2TMS	84.53

Table 4.14: Coefficient of variance for adapted microwave-assisted derivatives at 230 W

1.5 min	CV (%)	2.0 min	CV (%)	3.0 min	CV (%)	4.0 min	CV (%)
Mal 2TMS	4.41	Mal 2TMS	11.04	Ace 1TMS	5.71	Ace 1TMS	1.37
Phe 1TMS	6.59	Adi 2TMS	13.48	Pen 1TMS	8.63	Fum 2TMS	2.25
Adi 2TMS	6.62	Phe 1TMS	14.65	Fum 2TMS	8.82	Seb 2TMS	2.59
Suc 1M 1TMS c	8.52	Fum 2TMS	15.06	Adi 2TMS	9.09	Aco 3TMS	2.65
Fum 2TMS	8.76	Suc 1M 1TMS d	15.06	Pal 1TMS	9.27	Pen 1TMS	2.86
Aco 3TMS	8.76	Seb 2TMS	15.20	Ket 1M 2TMS a	9.73	Pal 1TMS	2.91
Suc 1M 1TMS d	9.17	Suc 1M 1TMS c	15.39	Seb 2TMS	10.26	Adi 2TMS	3.42
Pen 1TMS	9.52	Cit 4TMS	15.45	Ket 1M 2TMS b	10.41	Cit 4TMS	7.60
Suc 1M 1TMS b	9.75	Suc 1M 1TMS b	15.63	Suc 1M 1TMS c	10.85	Ket 1M 2TMS b	8.36
Ace 1TMS	9.78	Ket 1M 2TMS b	15.92	Suc 1M 1TMS a	11.13	Suc 1M 1TMS a	10.36
Seb 2TMS	9.93	Aco 3TMS	16.17	Aco 3TMS	11.38	Suc 1M 1TMS b	11.57
Pal 1TMS	10.19	Pal 1TMS	16.74	Cit 4TMS	11.43	Suc 1M 1TMS d	11.78
Suc 1M 1TMS a	10.79	Ace 1TMS	16.82	Suc 1M 1TMS b	11.53	Suc 1M 1TMS c	12.02
Ket 1M 2TMS a	11.09	Ket 1M 2TMS a	16.93	Suc 1M 1TMS d	11.80	Oro 3TMS	12.15
Ket 1M 2TMS b	13.24	Pen 1TMS	17.08	Cit 3TMS	12.59	Mal 2TMS	13.09
Cit 4TMS	13.52	Suc 1M 1TMS a	17.48	Oro 3TMS	15.44	Ket 1M 2TMS a	17.20
Oro 3TMS	15.39	Ace 2TMS	18.60	Phe 1TMS	16.73	Oxa 2TMS	21.96
Ace 2TMS	22.69	Oro 3TMS	20.57	Hip 1TMS	16.80	Ace 2TMS	27.68
Cit 3TMS	24.63	Cit 3TMS	22.00	Mal 2TMS	19.08	Phe 1TMS	31.58
Hip 1TMS	33.45	Hip 1TMS	31.33	Ace 2TMS	25.66	Mal 3TMS	49.67
Pyr 1M 1TMS	36.64	Oxa 2TMS	32.46	Mal 3TMS	28.25	Pyr 1M 1TMS	59.73
Oxa 2TMS	43.47	Hip 2TMS	33.01	Oxa 2TMS	36.36	Cit 3TMS	N/D
Hip 2TMS	44.67	Pyr 1M 1TMS	34.87	Hip 2TMS	41.86	Hip 1TMS	N/D
Mal 3TMS	63.65	Mal 3TMS	52.45	Pyr 1M 1TMS	75.50	Hip 2TMS	N/D

Table 4.15: Coefficient of variance for adapted microwave-assisted derivatives at 350 W

1.5 min	CV (%)	2.0 min	CV (%)	3.0 min	CV (%)	4.0 min	CV (%)
Ket 1M 2TMS a	7.89	Fum 2TMS	2.98	Fum 2TMS	2.45	Fum 2TMS	3.92
Oxa 2TMS	8.61	Ace 1TMS	3.24	Aco 3TMS	2.78	Ace 1TMS	4.33
Suc 1M 1TMS a	8.81	Suc 1M 1TMS a	3.51	Ace 1TMS	3.38	Pal 1TMS	4.77
Ace 1TMS	8.86	Suc 1M 1TMS d	3.52	Oro 3TMS	3.42	Seb 2TMS	4.87
Fum 2TMS	8.87	Suc 1M 1TMS b	3.78	Seb 2TMS	4.00	Ket 1M 2TMS a	5.93
Pen 1TMS	8.89	Suc 1M 1TMS c	3.95	Suc 1M 1TMS b	4.68	Suc 1M 1TMS a	6.84
Suc 1M 1TMS b	9.15	Pal 1TMS	4.17	Pen 1TMS	4.93	Aco 3TMS	7.02
Suc 1M 1TMS c	9.56	Seb 2TMS	4.54	Suc 1M 1TMS c	5.11	Ket 1M 2TMS b	7.07
Seb 2TMS	9.93	Ket 1M 2TMS b	4.70	Suc 1M 1TMS d	5.14	Suc 1M 1TMS d	7.18
Suc 1M 1TMS d	9.97	Aco 3TMS	6.20	Oxa 2TMS	5.51	Suc 1M 1TMS b	7.28
Ket 1M 2TMS b	10.14	Pen 1TMS	6.24	Pal 1TMS	5.84	Suc 1M 1TMS c	7.73
Aco 3TMS	10.39	Cit 3TMS	8.39	Suc 1M 1TMS a	6.03	Pen 1TMS	7.98
Pal 1TMS	11.21	Adi 2TMS	9.56	Cit 4TMS	6.99	Cit 3TMS	8.64
Phe 1TMS	12.97	Ket 1M 2TMS a	10.47	Adi 2TMS	7.78	Pyr 1M 1TMS	9.04
Pyr 1M 1TMS	13.11	Cit 4TMS	14.49	Ket 1M 2TMS a	7.86	Hip 1TMS	9.52
Adi 2TMS	13.66	Phe 1TMS	14.52	Ket 1M 2TMS b	8.46	Adi 2TMS	10.11
Hip 1TMS	16.10	Mal 2TMS	19.77	Mal 2TMS	10.87	Oxa 2TMS	11.71
Cit 3TMS	16.22	Hip 1TMS	22.14	Phe 1TMS	11.88	Cit 4TMS	15.57
Cit 4TMS	16.75	Oro 3TMS	23.37	Cit 3TMS	13.96	Mal 2TMS	17.86
Oro 3TMS	21.70	Pyr 1M 1TMS	23.99	Hip 1TMS	15.56	Phe 1TMS	18.84
Mal 2TMS	24.98	Oxa 2TMS	25.43	Ace 2TMS	31.72	Oro 3TMS	22.20
Mal 3TMS	63.32	Mal 3TMS	61.80	Pyr 1M 1TMS	43.26	Mal 3TMS	62.19
Ace 2TMS	N/D	Ace 2TMS	N/D	Mal 3TMS	47.08	Ace 2TMS	N/D
Hip 2TMS	N/D	Hip 2TMS	N/D	Hip 2TMS	N/D	Hip 2TMS	N/D

Table 4.16: Coefficient of variance for adapted microwave-assisted derivatives at 450 W

1.5 min	CV (%)	2.0 min	CV (%)	3.0 min	CV (%)	4.0 min	CV (%)
Adi 2TMS	1.00	Pal 1TMS	3.49	Adi 2TMS	2.38	Pal 1TMS	3.96
Fum 2TMS	1.90	Seb 2TMS	4.30	Pen 1TMS	2.69	Fum 2TMS	4.45
Aco 3TMS	1.94	Pen 1TMS	4.76	Aco 3TMS	2.99	Ace 1TMS	4.84
Seb 2TMS	2.03	Fum 2TMS	4.93	Pal 1TMS	3.06	Pen 1TMS	5.06
Pen 1TMS	2.56	Adi 2TMS	5.02	Oro 3TMS	3.34	Seb 2TMS	6.24
Pal 1TMS	2.84	Aco 3TMS	5.55	Suc 1M 1TMS a	3.40	Suc 1M 1TMS a	6.77
Ace 1TMS	3.67	Ace 1TMS	5.86	Seb 2TMS	3.61	Suc 1M 1TMS c	7.61
Ket 1M 2TMS a	6.97	Suc 1M 1TMS b	6.66	Fum 2TMS	3.67	Suc 1M 1TMS b	7.84
Suc 1M 1TMS a	7.04	Suc 1M 1TMS d	7.03	Phe 1TMS	3.94	Suc 1M 1TMS d	7.95
Suc 1M 1TMS d	7.24	Suc 1M 1TMS c	7.49	Suc 1M 1TMS d	4.13	Ket 1M 2TMS b	8.34
Suc 1M 1TMS b	7.75	Cit 4TMS	7.74	Suc 1M 1TMS c	4.35	Aco 3TMS	8.44
Oro 3TMS	7.84	Suc 1M 1TMS a	7.83	Suc 1M 1TMS b	4.35	Adi 2TMS	9.47
Suc 1M 1TMS c	8.05	Ket 1M 2TMS b	8.04	Oxa 2TMS	4.72	Ket 1M 2TMS a	9.62
Cit 4TMS	8.53	Oxa 2TMS	9.73	Ace 1TMS	4.77	Mal 2TMS	14.63
Ace 2TMS	11.51	Oro 3TMS	11.32	Ket 1M 2TMS b	6.15	Oxa 2TMS	15.02
Ket 1M 2TMS b	11.63	Phe 1TMS	12.70	Ace 2TMS	11.01	Cit 4TMS	15.26
Oxa 2TMS	12.70	Ket 1M 2TMS a	12.81	Ket 1M 2TMS a	11.55	Phe 1TMS	18.51
Mal 2TMS	13.81	Mal 2TMS	13.00	Mal 2TMS	12.17	Cit 3TMS	19.37
Phe 1TMS	26.93	Ace 2TMS	15.60	Cit 4TMS	14.43	Pyr 1M 1TMS	24.12
Mal 3TMS	53.58	Pyr 1M 1TMS	38.69	Mal 3TMS	28.20	Oro 3TMS	25.60
Pyr 1M 1TMS	66.17	Mal 3TMS	50.09	Pyr 1M 1TMS	35.31	Mal 3TMS	54.61
Cit 3TMS	N/D	Cit 3TMS	N/D	Cit 3TMS	N/D	Ace 2TMS	N/D
Hip 1TMS	N/D	Hip 1TMS	N/D	Hip 1TMS	N/D	Hip 1TMS	N/D
Hip 2TMS	N/D	Hip 2TMS	N/D	Hip 2TMS	N/D	Hip 2TMS	N/D

In Table 4.13 the microwave energy investigating 150 W, shows 21 derivatives with a CV ≤ 20% at a reaction time of 1.5 min; for 2.0 min, 19 derivatives; for 3.0 min, 21 derivatives and for 4.0 min, 17

derivatives had a CV value ≤ 20% respectively. Microwave energy of 230 W (Table 4.14) resulted in 17 derivatives with a CV ≤ 20% at 1.5 min; for 2.0 min, 17 derivatives; for 3.0 min, 19 derivatives and for 4.0 min, 16 derivatives had a CV value ≤ 20%. In Table 4.15 at 350 W, 1.5 min resulted in 19 derivatives with a CV ≤ 20%; for 2.0 min, 17 derivatives; for 3.0 min, 20 derivatives and for 4.0 min, 20 derivatives had a CV value ≤ 20%. Lastly at 450 W, Table 4.16 shows 18 derivatives with a CV ≤ 20%for the reaction times 1.5 and 4.0 min and 19 derivatives with a CV value ≤ 20% for 2.0 and 3.0 min.

The lowest investigated microwave energy (150 W) resulted in the largest number of derivatives with a CV ≤ 20% at 1.5 and 3.0 min. For 230 W, the largest number of derivatives had a CV ≤ 20% after derivatisation of 3.0 min. At 350 W, both 3.0 and 4.0 min of derivatisation resulted in the same number of derivatives with a CV ≤ 20%. The highest investigated microwave energy (450 W) resulted in the largest number of derivatives with a CV ≤ 20% at 2.0, 3.0 and 4.0 min. Overall the number of derivatives with a CV ≤ 20% are almost the same for the investigated microwave energies and between reaction times. Although it is a known fact that temperature and reaction time both have a significant effect on the derivatisation efficiency for organic acids (Moros *et al.*, 2017), these results demonstrate no pattern towards certain microwave energies nor reaction times, however individual derivatives will be focused on next to highlight some differences observed in Tables 4.13 - 4.16.

The monocarboxylic acid derivatives pentadecanoic acid 1TMS and palmitic acid 1TMS displayed repeatability with CV values ≤ 20%, but 3-phenylbutyric acid 1TMS showed CV values ranging from 6.48 to 31.58% (Tables 4.13 - 4.16). Tables 4.13 - 4.16 show that adipic acid 2TMS, fumaric acid 2TMS and sebacic acid 2TMS have CV values ≤ 20% at all conditions investigated. Oxalic acid 2TMS shows CV values that range from 5.51 to 43.47%, providing less variation for the higher microwave energies at 350 and 450 W regardless of the reaction time. Cis-aconitic acid 3TMS provided repeatable derivatisation with CV ≤ 20% at all adapted microwave-assisted conditions. Orotic acid consists of two keto- groups, but were not methoxymated as expected. The presence of the aromatic ring structure to which the keto- groups are bound to, might have an influence on the stability of the keto- groups. A tri- TMS derivative was formed when derivatising orotic acid. This may be due to silylation of the carboxyl and or the keto- or the amino groups. CV values range from 3.42 to 25.60% for this derivative. The keto- group of pyruvic acid was methoxymated, resulting in a derivative with only one TMS group, with CV values ranging from 9.04 to 75.50%, depicting a large variation range using different conditions.

More than one derivative was still observed for citric acid, hippuric acid, 2-ketoglutaric acid, malonic acid, N-acetyl-L-alanine and succinylacetone, following the addition of the methoxymation step and microwave-assisted derivatisation. In Tables 4.13 - 4.16 the CV for N-acetyl-L-alanine 1TMS is ≤

20% for all conditions. N-acetyl-L-alanine 2TMS showed a CV ≤ 20% for four conditions and was not detected at five conditions. Adapted microwave-assisted derivatisation formed two derivatives for citric acid at certain conditions. For citric acid 3TMS nine conditions show a CV ≤ 20% and citric acid 4TMS shows CV ≤ 20% for all conditions. Citric acid 4TMS was detected at all conditions, but citric acid 3TMS was only detected with twelve of the conditions. Compared to the CV values of citric acid 3TMS, lower values were obtained in the tetra- TMS form indicating better repeatability for citric acid 4TMS at all adapted microwave-assisted conditions.

As in Section 4.2.1, adapted microwave-assisted derivatisation also resulted in the formation of two hippuric acid derivatives at certain conditions. Hippuric acid 1TMS was formed at all conditions except at 230 W for 4.0 min and at 450 W for 1.5, 2.0, 3.0 and 4.0 min. Hippuric acid 2TMS was detected for some reaction times at 150 and 230 W. The mono- TMS derivative has a CV ≤ 20% for seven conditions and the di- TMS derivative has a CV > 20% for all conditions excluding 150 W for 3.0 min. The high CV values are possibly due to small peaks areas with a low signal to noise ratio as explained in Section 4.2.1. The instability of hippuric acid can be due to the fact that nitrogen consisting compounds are associated with unstable derivatives (Gerlo *et al.*, 2006) as experienced without the addition of methoxyamine aswell.

The compound 2-ketoglutaric acid resulted in two derivatives for all conditions (Tables 4.13 - 4.16). Methoxymation of the keto- group prevented cyclisation (Fiehn *et al.*, 2000) and keto- enol tautomerisation (Lai and Fiehn, 2016), but due to the syn and anti isoforms still resulted in two derivatives (Qiu and Reed, 2014). Tables 4.13 - 4.16 displayed CV values ≤ 20% at all conditions for both 2-ketoglutaric acid 1M 2TMS isoforms.

In Tables 4.13 - 4.16 the difference in derivatisation efficiency of malonic acid 2TMS and malonic acid 3TMS under adapted thermal derivatisation are displayed. All CV values for the di- TMS derivative of malonic acid are ≤ 20% except at 350 W for 1.5 min. The CV values for the tri- TMS derivative of malonic acid all go beyond 20% ranging from 28.20 to 64.08%. Results indicate that neither microwave energy nor reaction time influenced the derivatisation efficiency between the two derivatives of malonic acid. The four isomere derivatives for succinylacetone 1M 1TMS (a, b, c and d) resulted in CV values ≤ 20% for all conditions investigated.

Considering the drawbacks associated with obtaining multiple derivatives it is recommended to select derivatisation conditions which minimise the incidence of multiple derivatives (Bekele *et al.*, 2014). Although results showed that some conditions provided more repeatable derivatisation, the investigated conditions failed to eliminate formation of multiple derivatives for succinylacetone amongst other compounds. Another recommendation for quantitative analyses is to sum the

responses for multiple derivatives by assuming equal responses (Little, 2014). However, care should be taken when comparing response values for different compounds or derivatives since mass spectrometry may provide different responses for different derivatives (Fiehn, 2017). Therefore, only the derivatives with CV values ≤ 20% for a compound with multiple derivatives, will be used to compare derivatisation conditions in the next section.

In order to compare the effect of the pre-defined derivatisation conditions (Section 3.4) for each individual compound, scatter plots were constructed (Figs. 4.9 and B.1 - B.31). For compounds with more than one derivative, each derivative was plotted separately. All conditions in the scatter plots are distributed according to the CV (%) and average relative response (determined from the replicates) obtained within each condition. Keeping in mind that repeatability is the main performance criterion, derivatisation conditions with a CV ≤ 20% were considered to be repeatable.

4.2.5 Overview of compound specific derivatisation

In order to evaluate the effect of the pre-defined derivatisation conditions (Section 3.4) on each individual compound, scatter plots were constructed (Figs. 4.9 and B.1 - B.31) using CV (%) and average relative response (determined from five replicates) on the y- and x-axis, respectively. The plots include the derivatisation outcome as follows: thermal derivatisation (●), adapted thermal derivatisation (▲), microwave-assisted derivatisation (◆) and adapted microwave-assisted derivatisation (■) for all selected organic acids, at all temperature/ microwave energy and reaction time variations investigated. For compounds with multiple derivatives, each derivative was plotted separately. When assessing the plots, it is important to note that the scales of the x- and y-axes were determined according the lowest and highest values obtained for each derivative, which represents a focussed summary of the results that is unique for each scatter plot. The relative response distribution is divided into two groups, due to reduced instrument sensitivity for the analysis of microwave-assisted conditions (at 230 W for 1.5, 2.0 and 3.0 min and 450 W for 2.0, 3.0 and 4.0 min) and also for the adapted microwave-assisted conditions (at 150 W for 1.5 min; 230 W for 4.0 min; 350 W for 1.5, 2.0 and 4.0 min and 450 W for 1.5, 2.0, 3.0 and 4.0 min). The plots to follow are discussed in order of increasing complexity of functional groups, i.e. mono-, di-, tricarboxylic acids and additional functional groups. For the sake of flow, the above-mentioned parameters will not be repeated within the discussion of each scatter plot below, limiting the discussion to experimental findings and literature comparisons.

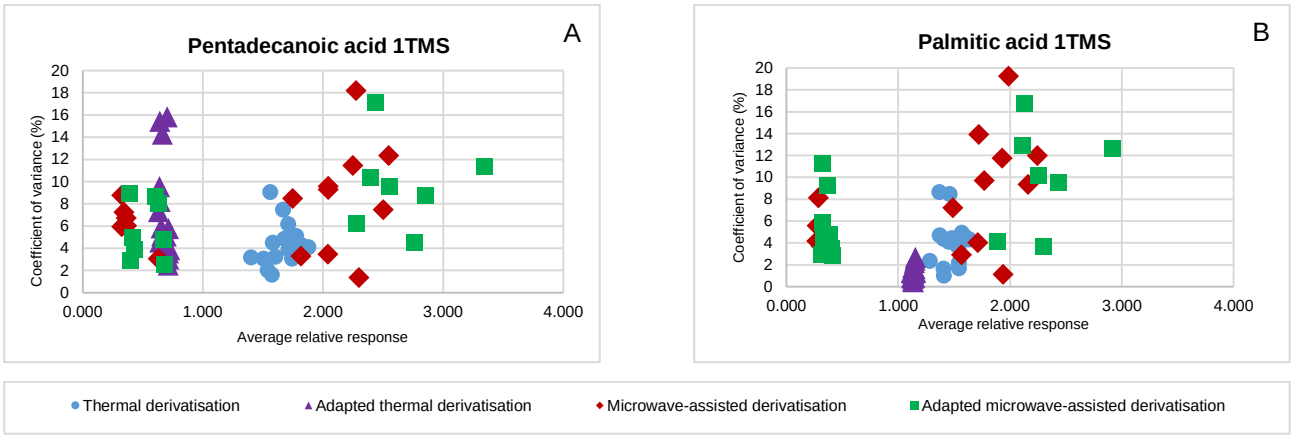


Figure 4.10: Compound derivatisation of monocarboxylic acids. Scatter plots of pentadecanoic acid (A) and palmitic acid (B). Thermal, adapted thermal, microwave-assisted and adapted microwave-assisted derivatisation outcome illustrated using CV (%) and average relative response results obtained within each condition.

The scatter plot of pentadecanoic acid 1TMS shows that all of the conditions within thermal, adapted thermal, microwave-assisted and adapted microwave-assisted derivatisation have a CV ≤ 20% (Fig. 4.10A). From the scatter plot of pentadecanoic acid 1TMS, grouping for thermal derivatisation is evident, supporting what was found in Section 4.2.1, with regards to multiple temperatures and reaction times resulting in similar results for this compound. Adapted thermal derivatisation also shows grouping of conditions, however the conditions separated (top left) from the main group do not indicate a pattern for similar temperatures or reaction times. The actual CV difference between the seemingly two groups are small (< 4%), which could easily be considered as one grouping. Microwave-assisted- and adapted microwave-assisted derivatisation resulted in the largest variation with the highest relative response values within the data presented in Figure 4.10A. High relative response values from microwave-assisted derivatisation was previously seen by Kouremenos *et al.* (2010), where microwave-assistance provided larger peak areas than conventional thermal derivatisation due to more efficient energy transfer from microwave-assistance. Even though the use of microwave energy resulted in higher relative response values for some conditions, the coefficient of variance suggests that microwave energy could not be used for derivatisation of pentadecanoic acid. From literature it is furthermore evident that microwave-assisted derivatisation efficiency can be influenced by the occurrence of unpredictable heating behaviour when more than one vial is derivatised at a time (Soderholm *et al.*, 2010), like the derivatisation procedure performed in this study. This may be seen as an argument to why varying CV values were achieved. Thermal derivatisation (Fig. 4.10A) resulted in the lowest CV values and relative response combination, suggesting that derivatisation with BSTFA-TMCS (99:1%) is sufficient when derivatising pentadecanoic acid 1TMS.

Palmitic acid 1TMS is also a monocarboxylic acid, but with an extended chain length (Table 2.3) and shows a similar scatter plot profile in Figure 4.10B to pentadecanoic acid 1TMS in Figure 4.10A,

however with a difference in the prominent grouping in the adapted thermal derivatisation conditions. When comparing the thermal and adapted thermal derivatisation outcome, the overall repeatability of both the relative response and CV values of the adapted methods, which included the use of methoxymation, outperforms thermal derivatisation. Literature speculates that the methoxymation heating temperature may improve derivatisation efficiency by increasing the solubility of compounds in pyridine (Gullberg *et al.*, 2004). This may explain why the derivatisation efficiency for palmitic acid is improved with the methoxymation step. However, the addition of a methoxymation step does not seem to have the same effect when microwave-assistance is used for derivatisation. The microwave results presented in Figure 4.10B shows more scattered values, suggesting more variation between conditions even though the CV values are still within the ≤ 20% benchmark. Unpredictable heating is a characteristic of derivatisation with microwave energy (Soderholm *et al.*, 2010), which might explain the results obtained. The possible advantage obtained with the use of methoxymation (in the adapted microwave-assistance method), is outperformed by the unpredictable microwave heating, resulting in variation prior to and after methoxymation. For palmitic acid adapted thermal derivatisation provided the condition with the lowest CV, thus best repeatability.

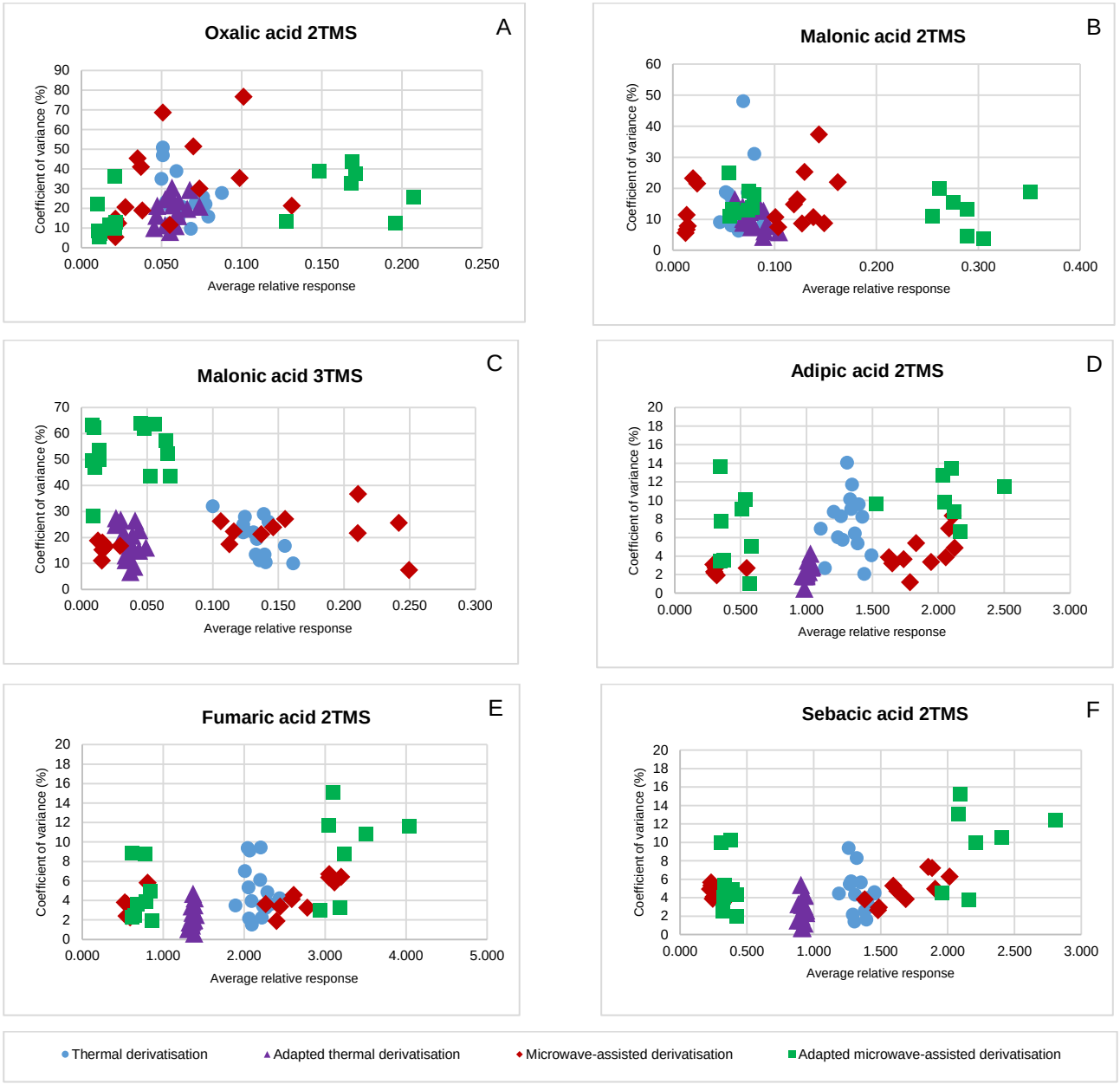


Figure 4.11: Compound derivatisation of dicarboxylic acids. Scatter plots of oxalic acid 2TMS (A), malonic acid 2TMS (B) and malonic acid 3TMS (C) adipic acid 2TMS (D), fumaric acid 2TMS (E), sebacic acid 2TMS (F). Thermal, adapted thermal, microwave-assisted and adapted microwave-assisted derivatisation outcome illustrated using CV (%) and average relative response results obtained within each condition.

From the investigated dicarboxylic acids in Figure 4.11, the compounds oxalic acid 2TMS (Fig. 4.11A), malonic acid 2TMS (Fig. 4.11B) and malonic acid 3TMS (Fig. 4.11C) displayed CV values > 20%. Oxalic acid and malonic acid are smaller dicarboxylic acids that are more volatile (Docherty and Ziemann, 2001). Especially oxalic acid resulted in reduced derivatisation efficiency, and less repeatable results as displayed in their scatter plots. The scatter plot for oxalic acid 2TMS (Fig. 4.11A) showed lower CV values for both the adapted thermal and adapted microwave-assistance

methods, when compared to the unadapted methods, showing that oxalic acid 2TMS benefits from the addition of a methoxymation step.

Malonic acid 2TMS (Fig. 4.11B) shows grouping of the results were obtained without the use of microwave-assisted derivatisation, highlighting the use of thermal (or adapted) derivatisation as a sufficient method. Pietrogrande *et al.* (2010) did report significant losses of malonic acid during the water evaporation step of an analytical standard sample, prior to the silylation step. As malonic acid is a low molecular weight compound that is volatile (Docherty and Ziemann, 2001), their findings seem plausible. From Figure 4.11C it is evident that adapted microwave-assisted derivatisation resulted in CV values much higher than the other three investigated derivatisation types for malonic acid 3TMS. Lastly, considering both CV and relative response, thermal derivatisation showed the best results for malonic acid 3TMS.

The derivatisation outcome of the remaining three dicarboxylic acids in Figure 4.11, adipic acid 2TMS (D), fumaric acid 2TMS (E), and sebacic acid 2TMS (F), show similar scatter plots and will be discussed collectively. Adapted thermal derivatisation made a significant difference to the derivatisation repeatability of these three derivatives with CV values < 6% achieved when using methoxyamine. Furthermore, thermal derivatisation resulted in higher relative response values, but also slightly higher CV values. Microwave-assisted and adapted microwave-assisted derivatisation showed similar results in the above-mentioned scatter plots, with the adapted method resulting in higher relative response values. A similar finding, with a higher relative response for adipic acid, was documented, following derivatisation with microwave-assistance and methoxyamine (Kouremenos *et al.*, 2010a). Overall, adapted thermal derivatisation provided the condition with the lowest CV values. However, conditions with higher CV can also be considered if a higher relative response and shorter derivatisation time are included as preferable criteria as long as the CV is ≤ 20%.

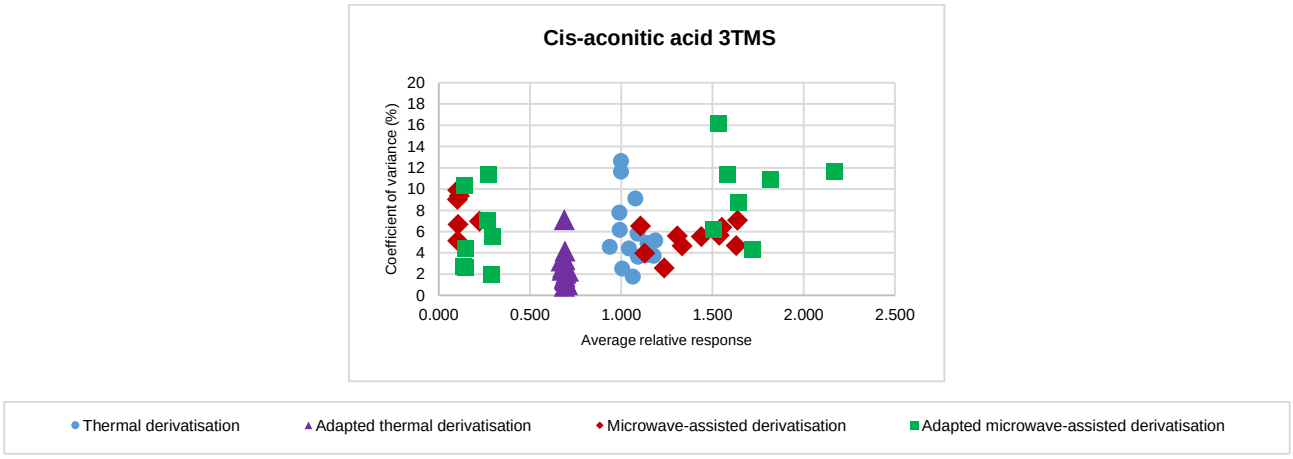


Figure 4.12: Compound derivatisation of a tricarboxylic acid. Scatter plot of cis-aconitic acid. Thermal, adapted thermal, microwave-assisted and adapted microwave-assisted derivatisation outcome illustrated using CV (%) and average relative response results obtained within each condition.

The scatter plot of cis-aconitic acid 3TMS shows that all derivatisation types resulted in CV ≤ 20% within all conditions (Fig. 4.12). Thermal and adapted thermal derivatisation shows prominent grouping, but with a few conditions separating from the norm. For thermal derivatisation the two conditions with CV > 10% represent 50°C for 30 min and 60°C for 60 min silylation, and for adapted thermal derivatisation the different condition with CV > 6% represents also 50°C for 30 min silylation. Interestingly this condition grouping differently within both thermal investigations, is the lowest temperature and shortest reaction time evaluated within this study. Irrespective of these low temperatures and short times, cis-aconitic acid was reported with a 6.3% CV following oxymation at 70°C for 30 min and BSTFA and TMCS derivatisation at 70°C for 60 min (Boulat *et al.*, 2003). The use of microwave energy, with and without methoxymation, resulted in greater relative response values for some investigated conditions, however with large variation between conditions. For cis-aconitic acid adapted thermal derivatisation resulted in the smallest relative response distribution and provided the condition with the highest repeatability, making the addition of a methoxymation step an important step to consider.

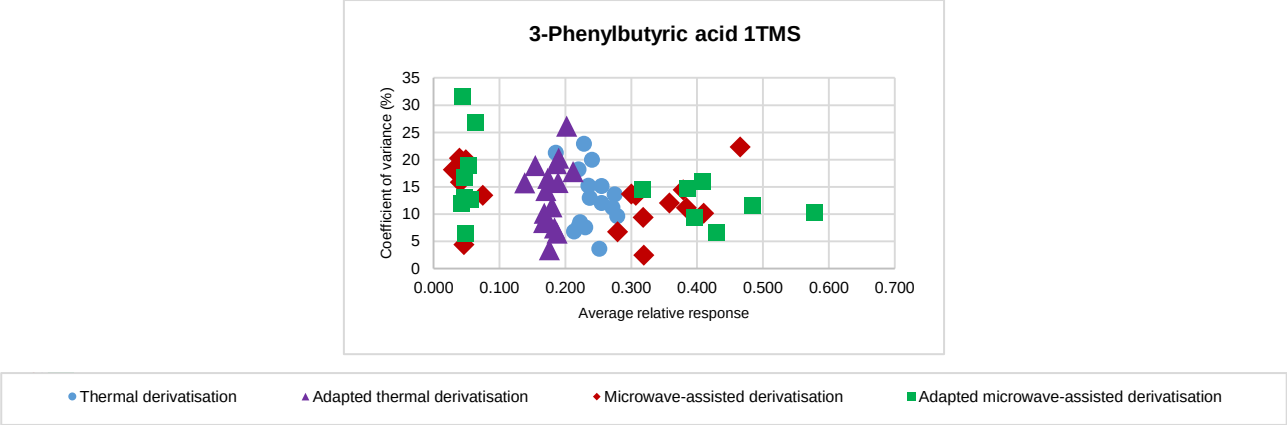


Figure 4.13: Compound derivatisation of a monocarboxylic acid with an aromatic ring. Scatter plot of 3-phenylbutyric acid. Thermal, adapted thermal, microwave-assisted and adapted microwave-assisted derivatisation outcome illustrated using CV (%) and average relative response results obtained within each condition.

In Figure 4.13 the results of 3-phenylbutyric acid following four types of derivatisation can be seen. This compound is a commonly used internal standard for GC-MS analysis of primarily organic acids (Reinecke *et al.*, 2012, Tran *et al.*, 2014), making it an important compound to include in a study like this. From the results obtained CV < 35% can be found for all conditions investigated. Looking at adapted thermal derivatisation, which showed the best grouping, the CV is distributed between 0 - 25% demonstrating that the different conditions have different effects on the derivatisation outcome of this compound. In general, it can be said that microwave-assisted derivatisation had the lowest CV values, which is interesting as this derivatisation type used the shortest reaction times. Considering the poor repeatability of 3-phenylbutyric acid obtained from this study, other internal standards should be considered as an internal standard for clinical work

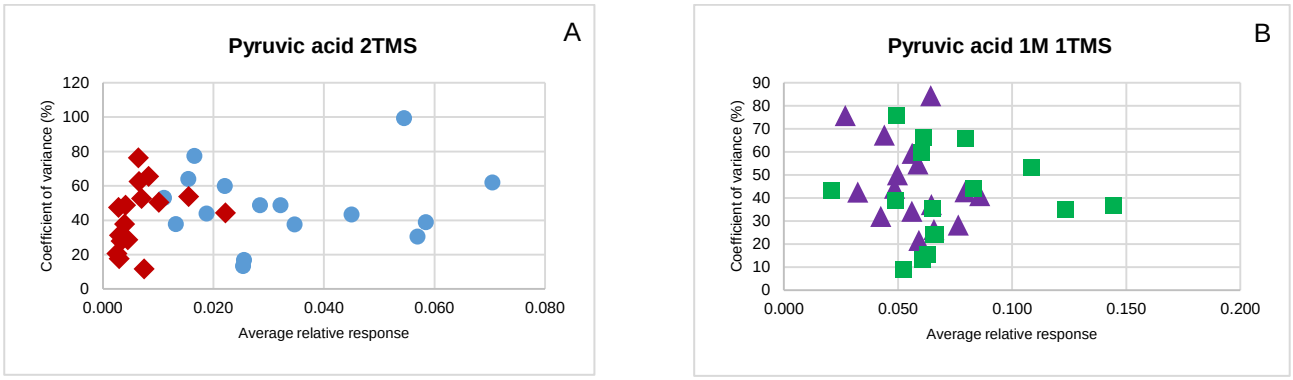


Figure 4.14: Compound derivatisation of a monocarboxylic acid with a keto- group. Scatter plots of pyruvic acid 2TMS (A) and pyruvic acid 1M 1TMS (B). Thermal, adapted thermal, microwave-assisted and adapted microwave-assisted derivatisation outcome illustrated using CV (%) and average relative response results obtained within each condition.

When derivatising pyruvic acid with BSTFA-TMCS (99:1%) with thermal- or microwave-assistance conditions, the scattered plot for pyruvic acid in the di- TMS (Fig. 4.13A) form was obtained and the addition of methoxyamine (with thermal or microwave energy) prior to silylation resulted in the pyruvic acid 1M (methoxymated) 1TMS form. For the four derivatisation types poor repeatability and low responses were obtained, suggesting compound loss. Further investigation implied pyruvic acid was probably evaporated when the aliquots were dried under a gentle stream of nitrogen in a heating block at 37°C (Section 3.2.3.1), since pyruvic acid is a volatile compound that is generally in a liquid form at room temperature (25°C) (Zeng *et al.*, 2018). As discussed in Chapter 2 (Fig. 2.6), silylation of pyruvic acid without methoxymation, may have resulted in two TMS derivatives due to keto- enol tautomerisation (Little, 2014). However, in this study pyruvic acid was only detected in its di- TMS form when using thermal- and microwave-assisted derivatisation conditions. This could be due to the reduced concentration of pyruvic acid and does not necessarily imply the formation of only the di- TMS derivative at the investigated conditions. The addition of methoxymation is recommended by literature for compounds with keto- groups, to reduce the number of derivatives obtained with silylation (Kumari *et al.*, 2011). However, if the same derivatives are produced repeatably following silylation, a compound like pyruvic acid might not need methoxymation. In Figure 4.14A, poor repeatability was obtained for pyruvic acid, with CV > 20% for most of the thermal and microwave-assisted derivatisation conditions investigated. The microwave-assisted derivatisation results did show some grouping, but with low relative response values.

In Figure 4.14B the results of pyruvic acid following methoxymation is evident, showing a methoxymated mono- TMS form thereof, and no results were obtained for this derivative without methoxymation. When the keto- group of pyruvic acid is methoxymated, only the carboxyl group can be silylated, resulting only in a 1M 1TMS derivative as discussed in Section 2.4.3.1. The difference in Figures 4.14A and 4.14B is the absence or presence of methoxymation, as performed for all adapted conditions (Fig. 4.14B) at 30°C for 90 min. Here the CV also resulted in values higher than

20% for most conditions within adapted thermal- and adapted microwave-assisted derivatisation. For pyruvic acid the methoxymation process does not seems like a good option. According to Bekele *et al.* (2014), silylation temperature has a negligible effect on the derivatisation efficiency for pyruvic acid. However for more efficient methoxymation of pyruvic acid it is suggested to use either a high temperature (50°C) for a short reaction time (30 min) or low temperature (30°C) for a long reaction time (60 min) (Bekele *et al.*, 2014), which does not correspond to the methoxymation condition at 30°C for 90 min used in this study based on work from Abbiss *et al.* (2015) and Fiehn (2017). Irrespective of using a methoxymation step, Figure 4.14B showed poor repeatability for pyruvic acid.

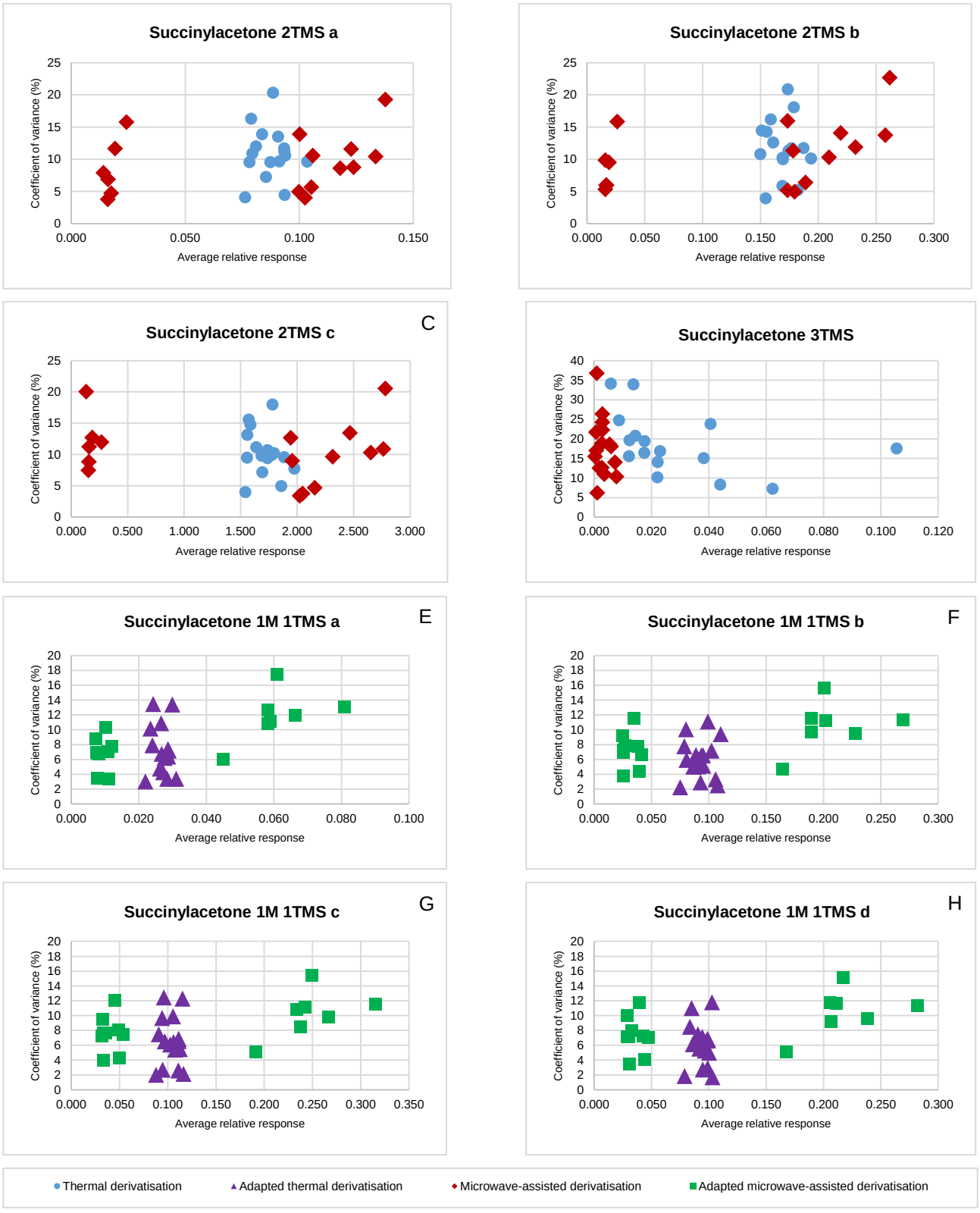


Figure 4.15: Compound derivatisation of a monocarboxylic acid with two keto- groups. Scatter plots of succinylacetone 2TMS a (A), succinylacetone 2TMS b (B), succinylacetone 2TMS c (C), succinylacetone 3TMS (D), succinylacetone 1M 1TMS a (E), succinylacetone 1M 1TMS b (F), succinylacetone 1M 1TMS c (G) and succinylacetone 1M 1TMS d (H). Thermal, adapted thermal, microwave-assisted and adapted microwave-assisted derivatisation outcome illustrated using CV (%) and average relative response results obtained within each condition.

When silylating succinylacetone with N,O-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane [BSTFA-TMCS (99:1%)] with thermal- or microwave-assistance conditions, the derivatives revealed in the scatter plots of Figures 4.15A - 4.15D were obtained. Considering the structure of succinylacetone (Table 2.3) there are three possible functional groups that can be silylated (Kouremenos *et al.*, 2010a, Little, 2014). Since succinylacetone contains two keto- groups it was expected that multiple derivatives for this compound would be present as discussed in Section 2.4.3.1 (Qiu and Reed, 2014). In addition, Qiu and Reed (2014) also explains that the number of derivatives may increase due to the formation of syn and anti isoforms. The three isomers of succinylacetone 2TMS showed similar results and CV < 25% were obtained for all thermal- and microwave-assisted derivatisation conditions investigated (Figs. 4.15A - 4.15C). The grouping of the results obtained by thermal derivatisation are more prominent, suggesting that the investigated conditions had similar effects on the derivatisation efficiency of succinylacetone 2TMS. From the succinylacetone 3TMS (Fig. 4.15D) result, microwave-assisted conditions resulted in better repeatability than thermal derivatisation, with lower CV values, except for the condition at 230 W for 3.0 min.

The addition of methoxyamine prior to silylation (thermal or with microwave-assistance) resulted in the derivatives revealed in Figures 4.15E - 4.15H. Although the structure of succinylacetone contain two keto- groups, only one was methoxymated at a time, forming syn and anti isoforms (Qiu and Reed, 2014). Only one keto- group was probably methoxymated at a time due to sterical hindrance that would also explain why a 1M 2TMS derivative was not detected. The methoxyamine treated succinylacetone resulted in four mono- TMS derivatives (Figs. 4.15E - 4.15H). Again, similar results for isoforms are evident within all figures displaying a CV ≤ 20% and the best repeatability for adapted thermal derivatisation. The adapted microwave-assisted derivatisation results show that divided grouping is visible between conditions. The same conditions are divided in the same pattern between the derivatives. Furthermore, multiple succinylacetone derivatives have been previously reported in literature where derivatisation with oxymation, instead of methoxymation, and silylation resulted in four succinylacetone derivatives with intra- and interday CV values ≤ 20% (Zhou *et al.*, 2016). Although methoxymation did not reduce the number of derivatives formed for succinylacetone, the results from adapted thermal- and adapted microwave-assisted derivatisation show that methoxymation improved reproducibility, as Figures 4.15E - 4.15H show CV ≤ 20% for all four derivatives.

A B

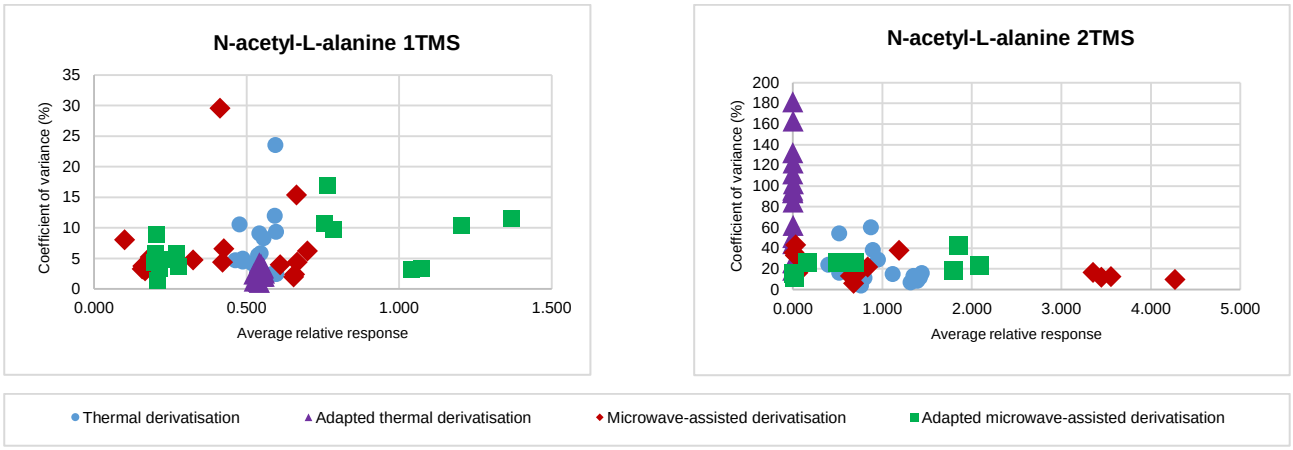


Figure 4.16: Compound derivatisation of a monocarboxylic acid with a keto- and amino group. Scatter plots of N-acetyl-L-alanine 1TMS (A) and N-acetyl-L-alanine 2TMS (B). Thermal, adapted thermal, microwave-assisted and adapted microwave-assisted derivatisation outcome illustrated using CV (%) and average relative response results obtained within each condition.

Derivatisation of N-acetyl-L-alanine resulted in a mono- TMS (Fig. 4.16A) and a di- TMS (Fig. 4.16B) derivative within all four derivatisation types investigated, but the di- TMS derivative was not detected within the microwave-assisted condition at 450 W for 3.0 min and adapted microwave-assisted conditions at 150 W for 1.5 min; 350 W for 1.5, 2.0 and 4.0 min and 450 W for 4.0 min. These parameters preventing the detection of the di- TMS derivative are random with no increase/ decrease in temperature or microwave energy. These conditions are also the conditions mentioned above with lower response values due to reduced instrument sensitivity. The occurrence of low response values for several derivatives could be corrected by normalising the data with the internal standard (C19:0Me) except for derivatives such as N-acetyl-alanine 2TMS which already had a low response value and reduced sensitivity which results in a zero value to be normalised.

The outcome of these two derivatives are in accordance to work reported by Gerlo *et al.* (2006), where N-acetylated amino acids, derivatised with BSTFA-TMCS (99:1%) resulted in the formation of two derivatives, favouring a longer reaction time for the formation of the di- TMS form. For N-acetyl-L-alanine 1TMS a CV ≤ 20% was obtained for all conditions investigated except for two represented by thermal derivatisation at 70°C for 120 min and, microwave-assisted derivatisation at 350 W for 2.0 min. The mono- TMS derivative of N-acetyl-L-alanine shows good repeatability following adapted thermal derivatisation with CV values < 5% for all conditions investigated (Fig. 4.16A). This outcome advises that when N-acetyl-L-alanine is a compound of interest, methoxymation could be advantageous as a derivatisation step. The same is not true when evaluating the results from the N-acetyl-L-alanine 2TMS derivative, where adapted thermal derivatisation resulted in very low relative responses with very high CV values (Fig. 4.16B). As discussed in Section 4.2.1.2, low relative responses may cause high CV values due to a low signal-to-noise ratio and inconsistent integration (Amigo *et al.*, 2008). Assessment of the other

derivatisation types i.e. thermal-, microwave-assisted- and adapted microwave-assisted derivatisation also resulted in lower CV values for N-acetyl-L-alanine 1TMS than for the di- TMS derivative.

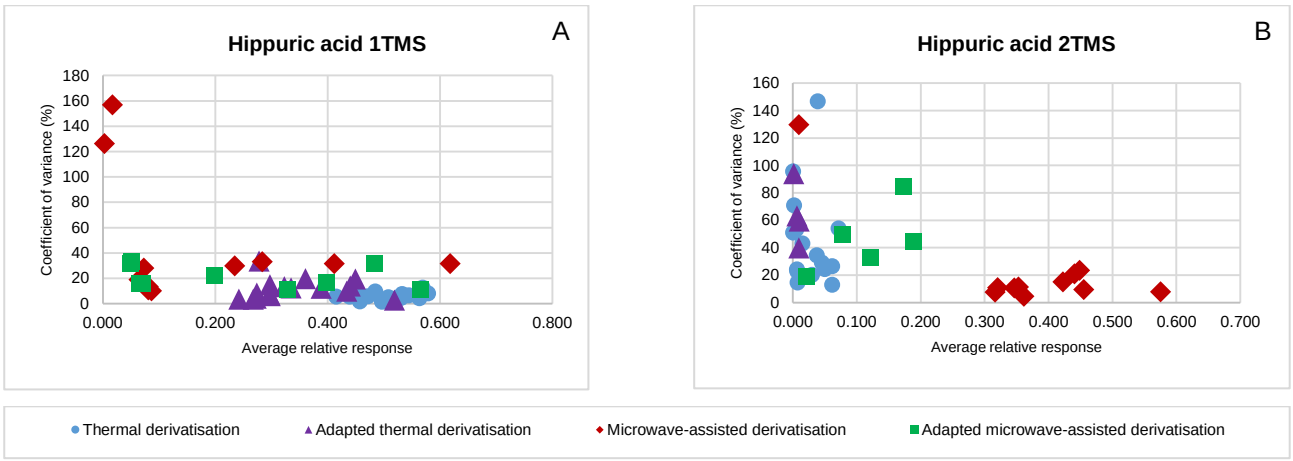


Figure 4.17: Compound derivatisation of a monocarboxylic acid with an aromatic ring, keto- and amino group. Scatter plots of hippuric acid 1TMS (A) and hippuric acid 2TMS (B). Thermal, adapted thermal, microwave-assisted and adapted microwave-assisted derivatisation outcome illustrated using CV (%) and average relative response results obtained within each condition.

The formation of two derivatives for hippuric acid (Figs. 4.17A and 4.17B) is a common occurrence as explained in Section 4.2.1.2 and detected within thermal-, adapted thermal-, microwave-assisted- and adapted microwave-assisted conditions. When using thermal derivatisation hippuric acid resulted in two derivatives at all conditions, howeveronly one derivative was formed when using adapted thermal- [conditions: 50°C (30, 60, 90 and 120 min); 70°C (30, 60, 90 and 120 min) and 85°C (30, 60, 90 and 120 min)], microwave-assisted- [conditions: 150 W (3.0 and 4.0 min); 230 W (1.5, 2.0, 3.0 and 4.0 min); 350 W (1.5 min) and 450 W (2.0, 3.0 and 4.0 min)] and adapted microwave-assisted derivatisation [conditions: 150 W (1.5 min) and 350 W (1.5, 2.0, 3.0 and 4.0 min)]. Then again, when using adapted microwave-assisted derivatisation at 450 W for 1.5, 2.0, 3.0 and 4.0 min, no derivatives for hippuric acid was formed (explaining the limited green square in the figures above). The formation of multiple derivatives is considered a derivatisation drawback, resulting in devided peak abundances as discussed in Section 2.4.2. Conditions leading to the formation of only one derivative is thus more preferable. However, the investigated conditions that have reduced the number of derivatives from two to one corresponds with most conditions associated with reduced instrument sensitivity (first paragraph of this section), except for all the adapted thermal conditions. Apart from the fact that the response of hippuric acid was reduced due to multiple derivatives, gives hippuric acid usually a low response (Gerlo *et al.*, 2016) as explained in Section 4.2.2.2. Along with reduced instrument sensitivity, the response was so low that no value was obtained that could be normalised.

The outcome of this two derivatives are in accordance with work reported by Wachsmuth *et al.* (2015), where hippuric acid was derivatised with methoxymation (60°C for 60 min) and silylated with N-methyl-(trimethylsilyl)trifluoroacetamide (MSTFA) at 60°C for 60 min. The mono- TMS derivative of hippuric acid (Fig. 4.17A) displays lower CV values for thermal-, adapted thermal- and adapted microwave-assisted derivatisation, when compared to hippuric acid 2TMS (Fig. 4.17B). Contradictory microwave-assisted derivatisation shows lower CV values for hippuric acid 2TMS than hippuric acid 1TMS. For hippuric acid the mono- TMS derivative shows more repeatable derivatisation for the thermal derivatisation type. The two microwave-assisted conditions in Figure 4.17A with very high CV values are both at 150 W for 1.5 min (> 120%) and 2.0 min (> 150%) respectively. In the scatter plot for the di- TMS derivative the two conditions with the highest CV are associated with thermal derivatisation (85°C for 120 min) and microwave-assisted derivatisation (450 W for 2.0 min). In both scatter plots it can be seen that the addition of methoxymation did not improve the derivatisation outcome of hippuric acid.

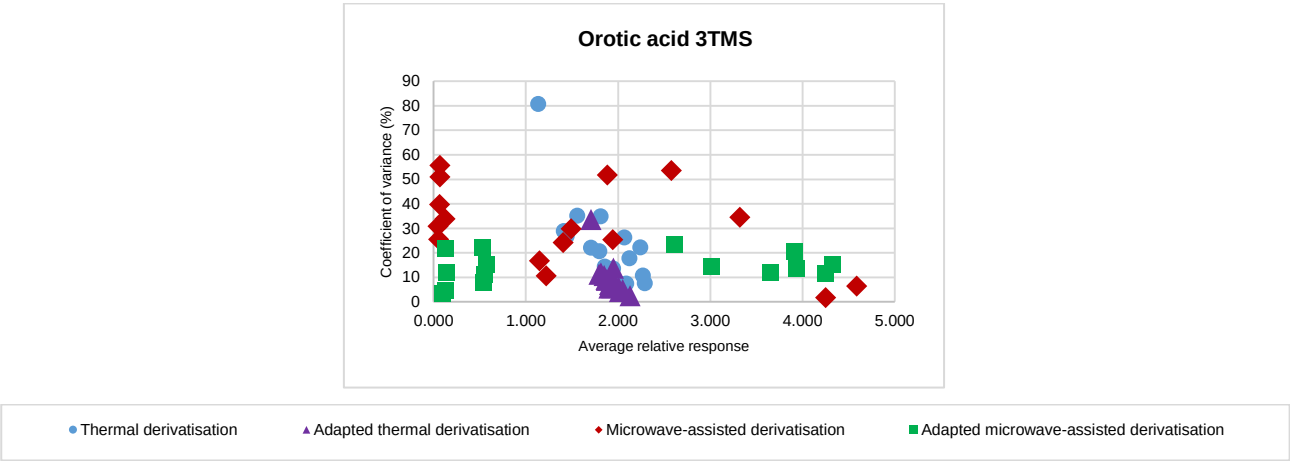


Figure 4.18: Compound derivatisation of a monocarboxylic acid with two keto- and two amino groups. Scatter plot of orotic acid 3TMS. Thermal, adapted thermal, microwave-assisted and adapted microwave-assisted derivatisation outcome illustrated using CV (%) and average relative response results obtained within each condition.

The scatter plot representing the derivatisation outcome of orotic acid 3TMS (Fig. 4.18) shows the highest CV values within thermal and adapted thermal derivatisation were both obtained at the lowest temperature (50°C) and shortest reaction time (30 min). Treating the sample with methoxyamine lead to more repeatable results, as shown by the adapted thermal derivatisation results, with CV ≤ 20% obtained for all conditions except the one investigating 50°C for 30 min. From literature the use of methoxymation is not commonly associated with orotic acid. However, other derivatisation methods are often added to investigate this compound as seen in a study by Kumari *et al.* (2015), where oxymation (60° for 30 min) and BSTFA with TMCS (80°C for 10 min) was used as derivatisation method, resulting in 5.1% intraday and 4.8% interday repeatability. Also the use of MSTFA with TMCS at 60°C for 60 min was reported as derivatisation method for urine analysis

investigating orotic acid (Wojtowicz *et al.*, 2010). The adapted microwave-assisted method shows great relative response distribution and CV < 25%. Then again microwave-assisted derivatisation resulted in varying results with the largest number of conditions investigated showing CV > 20%.

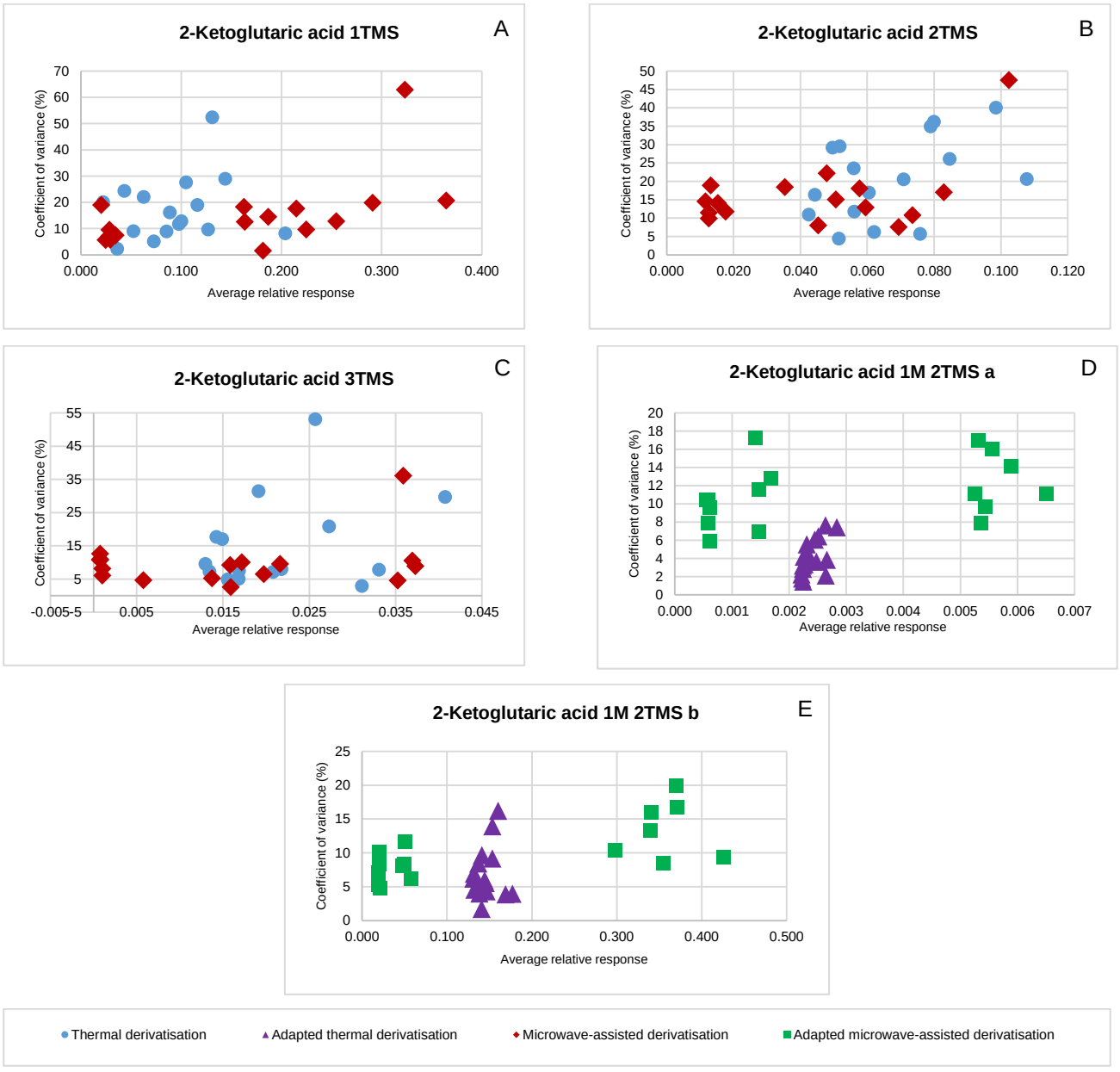


Figure 4.19: Compound derivatisation of a dicarboxylic acid with two keto- groups. Scatter plots of 2-ketoglutaric acid 1TMS (A), 2-ketoglutaric acid 2TMS (B), 2-ketoglutaric acid 3TMS (C), 2-ketoglutaric acid 1M 2TMS a (D) and 2-ketoglutaric acid 1M 2TMS b (E). Thermal, adapted thermal, microwave-assisted and adapted microwave-assisted derivatisation outcome illustrated using CV (%) and average relative response results obtained within each condition.

Derivatisation of 2-ketoglutaric acid for the thermal and microwave-assisted methods resulted in three derivatives for this compound as seen in Figures 4.19 - A) 1TMS; B) 2TMS and C) 3TMS. As discussed in Section 4.2, this is possible considering the structure of 2-ketoglutaric acid which contains two carboxyl groups and one keto- group. Based on the overall results from Figures 4.19A

- 4.19C, microwave-assisted derivatisation resulted in the largest amount of investigated conditions with a CV ≤ 20%. One condition in each microwave-assisted result contained a high CV value represented by 350 W for 3.0 min with CV > 60% (Fig. 4.19A); CV > 45% (Fig. 4.19B) and CV > 30% (Fig. 4.19C). The addition of methoxyamine to 2-ketoglutaric acid resulted in Figures 4.19D and 4.19E, representing 1 methoxymated (1M) 2TMS a, and 1M 2TMS b respectively, due to syn and anti isoforms (Qiu and Reed, 2014). The results support the use of adapted thermal derivatisation to ensure a more repeatable derivatisation outcome as CV < 10% (Fig. 4.19D) and CV < 20% (Fig. 4.19E) were obtained for all conditions investigated. The effect of methoxymation has also been demonstrated in literature were the derivatisation efficiency of 2-ketoglutaric acid with a longer reaction time (17 hours) and lower temperature (20°C) was found favourable when optimising derivatisation conditions (Gullberg *et al.*, 2004). Considering that repeatability is the main performance criterion adapted thermal is the derivatisation type to consider for 2-ketoglutaric acid.

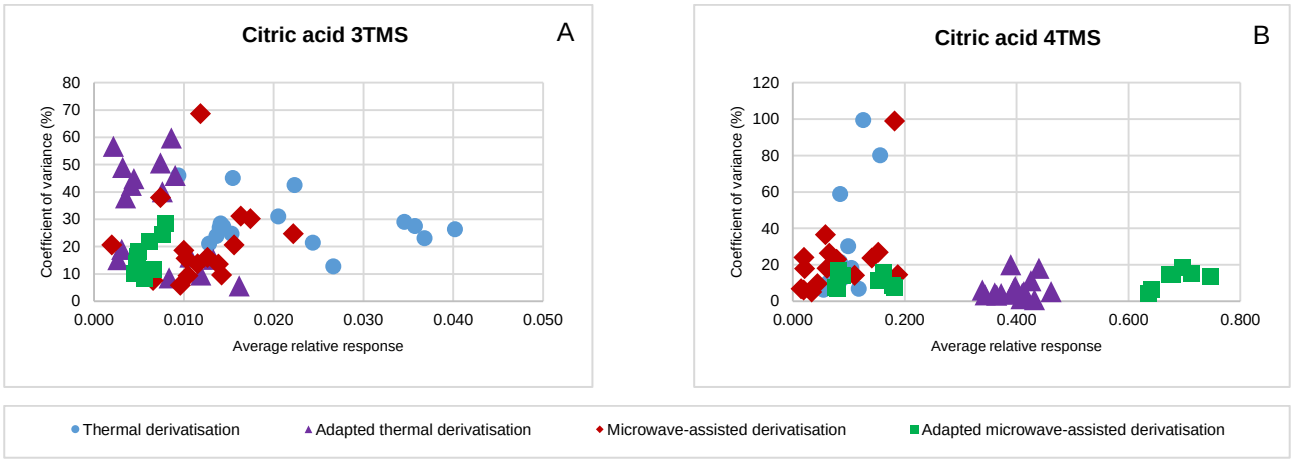


Figure 4.20: Compound derivatisation of a tricarboxylic acid with a hydroxyl group. Scatter plots of citric acid 3TMS (A) and citric acid 4TMS (B). Thermal, adapted thermal, microwave-assisted and adapted microwave-assisted derivatisation outcome illustrated using CV (%) and average relative response results obtained within each condition.

Following derivatisation of citric acid using all four types, a tri- TMS (Fig. 4.20A) and a tetra- TMS (Fig. 4.20B) derivative were obtained (Section 4.2.1). The derivatisation outcome for these two derivatives show prominent differences when compared to each other. Citric acid 3TMS shows the best repeatability for adapted microwave-assisted derivatisation, while adapted thermal derivatisation shows this for citric acid 4TMS. Figure 4.20B shows CV ≤ 20% for all adapted thermal- and adapted microwave-assisted conditions, indicating that the addition of the methoxymation process improves derivatisation repeatability for citric acid 4TMS. Although methoxymation is not usually performed for citric acid derivatisation considering the lack of a keto- or aldehyde groups, oxymation at 70°C for 30 min followed by BSTFA and TMCS derivatisation at 70°C for 60 min resulted in a 19.6% CV in a previous study (Boulat *et al.*, 2003). This study demonstrates that there is some benefit to adding other derivatisation types to typically used derivatisation reagents. This is

also prominent in literature where an array of microwave energies and reaction times are performed when a metabolite like citric acid is derivatised (Silva and Ferraz, 2004, Kouremenos *et al.*, 2010a). In both figures above, the one adapted microwave-assisted derivatisation condition shown by the two derivatives of citric acid (towards the top of the figures), represents 350 W for 3.0 min with CV values of 68.70% (Fig. 4.20A) and 99.02% (Fig. 4.20B). Only citric acid 4TMS was detected when using the adapted microwave-assisted conditions at 230 W for 4.0 and 450 W for 1.5, 2.0 and 3.0 min. All these conditions were analysed with reduced instrument sensitivity and affected only citric acid 3TMS with low response values, resulting in zero abundance values which could not be normalised (Tables 4.14 - 4.16).

4.2.6 Overview of sample derivatisation

For the final comparison the derivatisation type and condition resulting in the best repeatability for the investigated organic acids (excluding hippuric-, oxalic-, malonic- and pyruvic acid as discussed in Section 4.2.5) was used to construct a cumulative distribution graph (Fig. 4.21). Results represent adipic acid, cis-aconitic acid, citric acid, fumaric acid, 2-ketoglutaric acid, orotic acid, palmitic acid, pentadecanoic acid, 3-phenylbutyric acid, sebacic acid and succinylacetone investigated with thermal derivatisation (T 70°C for 90 min); adapted thermal derivatisation (AT 60°C for 30 min); microwave-assisted derivatisation (M 150 W for 1.5 min) and adapted microwave-assisted derivatisation (AM 350 W for 3.0 min) expressed as CV (%) values. In Appendix B the CV calculated results of the 12 organic acids (as discussed in Section 4.2.5 hippuric-, oxalic-, malonic- and pyruvic acid was not included within this calculation to prevent the skewing of results) were determined using five replicates analysed within each investigated derivatisation condition. This CV value was expressed as a percentage (x-axis) against the number of organic acids analysed also using percentage as a measuring unit (y-axis). Figure B.1.1 contains the findings of thermal derivatisation (temperature and reaction time); Figure B.1.2 illustrates the results of adapted thermal derivatisation (temperature and reaction time); in Figure B.1.3 the results of microwave-assisted derivatisation (watt and reaction time) can be found and in Figure B.1.4 adapted microwave-assisted derivatisation (watt and reaction time) is shown. Based on these findings the specific derivatisation conditions with the lowest CV value within each type of derivatisation is shown in Figure 4.21.

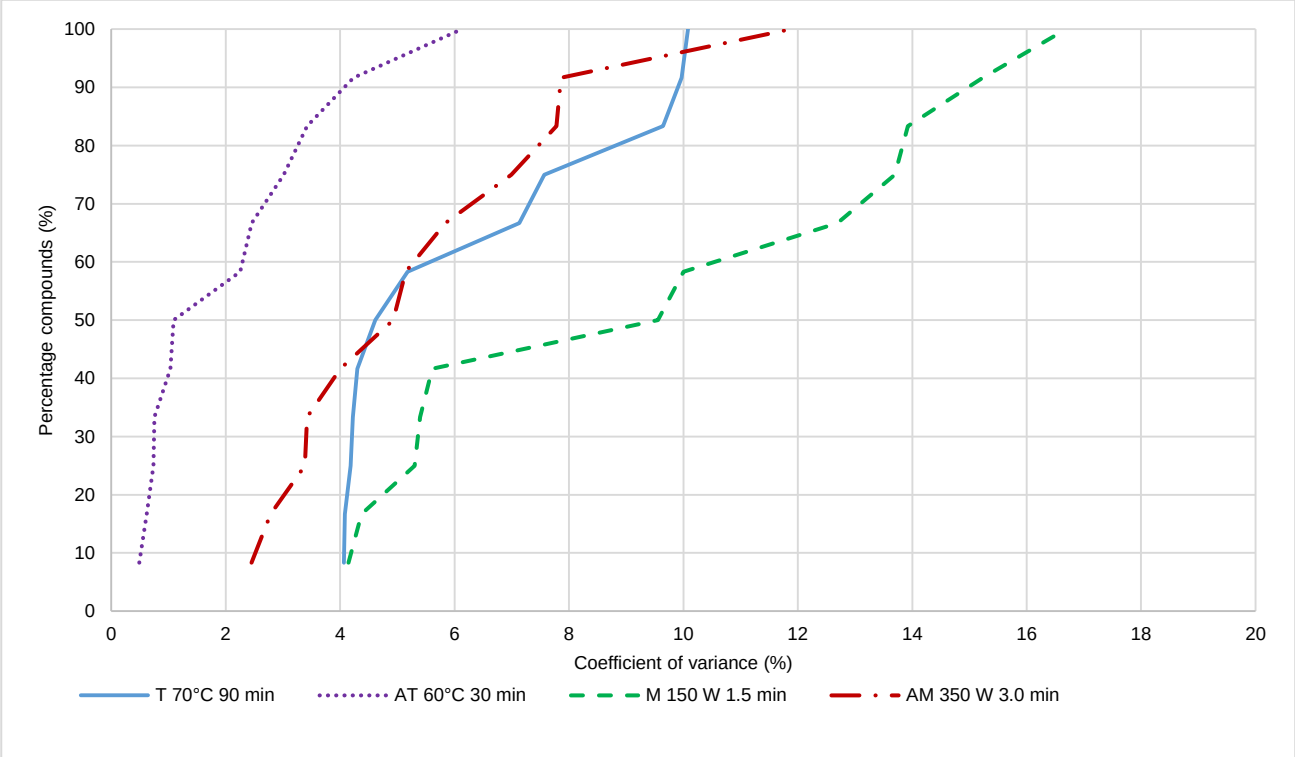


Figure 4.21: Cumulative distribution plot of most repeatable derivatisation conditions within each derivatisation type. The amount of organic acids (%) expresed against the CV calculated for the organic acids after thermal- (T) at 70ºC for 90 min; adapted thermal- (AT) at 60°C for 30 min; microwave-assisted (M) at 150 W for 1.5 min and adapted microwave-assisted derivatisation (AM) at 350 W for 3.0 min.

As seen in Figure 4.21 adapted thermal derivatisation at 60ºC for 30 min performed better than the other types of derivatisation, when considering reproducibility as a performance criterion. It is clear that all of the 12 organic acids (100%) achieved a CV value of < 7% when using these conditions for adapted thermal derivatisation. Thermal derivatisation at 70°C for 90 min had a CV < 11%, microwave-assisted derivatisation at 150 W for 1.5 min had a CV < 12% and adapted microwave-assisted derivatisation at 350 W for 3.0 min had a CV of < 17% when considering 100% of the selected organic acids. In Figure 4.21 it can be seen that it is possible to derivatise the 12 organic acids simultaneously using any of the four derivation types with any of the four conditions mentioned to achieve repeatability with a CV value of ≤ 20%. In practice, a universal derivatisation type with fixed conditions are applied for organic acids; it is clear that individual organic acids perform differently at specific conditions supporting the aim to compare parameters per compound and not in general. The type and condition most optimal for all compounds of interest should be implemented with known limitations.

CHAPTER 5: CONCLUSION

5.1 Introduction

Derivatisation is a technique used to transform the chemical characteristics of compounds in order to improve detectability in gas chromatography (Orata, 2012). Silylation, a type of derivatisation, is commonly used to improve the detection of organic acids with gas chromatography-mass spectrometry (GC-MS) (Christou *et al.*, 2014, Gallagher *et al.*, 2018), through the substitution of an active hydrogen with a trimethylsilyl (TMS) group (Husek, 2000). Organic acids are very polar and thermally unstable compounds making them generally unsuitable for gas chromatography analysis without derivatisation (Kaluzna-Czaplińska, 2011). Furthermore organic acids consist of at least one carboxyl group and other functional groups such as hydroxyl and keto- groups (Rinaldo, 2008). Analysis of organic acids enables an array of clinical assessments, deeming it crucial to investigate the procedures to which organic acids are subjected to prior GC-MS analysis (Chapter 1). Although protocols for organic acid analysis by GC-MS can easily be obtained in literature, established protocols or standard methods for reliable organic acid derivatisation are somewhat lacking, as demonstrated by the literature overview in Chapter 2. Pre-defined derivatisation parameters required for both, thermal- and microwave-assisted derivatisation procedures, were evaluated in this study (Chapter 3). By focusing on repeatability determined from coefficient of variance (CV) results, Chapter 4 comprises of all the results obtained following sample preparation, GC-MS analysis and statistical comparisons. The conclusions of this study are presented in Chapter 5, and summarises the findings and lastly recommendations are made to guide future work aimed at repeatable derivatisation procedures for organic acids.

5.2 General conclusion

In the first part of this study, the selection of parameter increments to be investigated (i.e. temperatures, microwave energies and reaction times) were focused on, using literature-based studies as stipulated by the **first objective**. From literature it is evident that derivatisation conditions generally used vary between studies. The selection of parameter increments was based on the literature conditions within ranges of 50 - 85°C and 30 - 90 min for both thermal derivatisation types; and 150 - 350 W and 1.5 - 4.0 min for both microwave-assisted derivatisation types. The results from this study supports the overall use of these conditions for thermal-, adapted thermal, microwave-assisted and adapted microwave-assisted derivatisation as acceptable repeatability was achieved within these ranges. No clear trends were observed with regards to increased (or decreased) temperature or microwave energy and longer (or shorter) reaction times, making it possible to

conclude that these parameter increments or even the parameters investigated are not the only factors to consider when evaluating acceptable repeatability.

As set out by the **second objective,** parameters within specific conditions for thermal-, adapted thermal-, microwave-assisted- and adapted microwave-assisted derivatisation types were compared for selected individual organic acids. All together, no inclination favouring specific derivatisation temperatures, microwave energies or reaction times were observed when evaluating the results. The findings do however demonstrate that the individual organic acids perform different, with derivatisation efficiencies largely influenced by the structure of the compound, with better repeatability seen when derivatising only carboxyl functional groups. From all conditions investigated, 60°C for 30 min was identified as the condition within adapted thermal derivatisation to provide the least variation for the included organic acids.

The results obtained using the selected derivatisation temperatures, microwave energies and reaction times, helped to achieve the **third objective** to compare repeatability within and between conditions for thermal-, adapted thermal-, microwave-assisted- and adapted microwave-assisted derivatisation types for selected individual organic acid derivatives. Thermal derivatisation resulted in better repeatability for pentadecanoic acid, but for palmitic acid, adipic acid, fumaric acid, sebacic acid and cis-aconitic acid better repeatability was found when using adapted thermal derivatisation. Although oxalic acid and malonic acid, (both dicarboxylic acids without other functional groups) showed better repeatability when using adapted thermal derivatisation, parameter selection should be done with caution since changes in the parameter intervals or the exactness of the intervals will greatly influence repeatability. Malonic acid was the only dicarboxylic acid with no other functional group that formed two TMS derivatives.

The organic acids, 3-phenylbutyric acid, pyruvic acid and orotic acid, resulted in only one derivative per derivatisation type investigated. Between the derivatisation types, adapted thermal derivatisation displayed better repeatability for 3-phenylbutyric acid and orotic acid. Poor repeatability was obtained for pyruvic acid for all derivatisation types. Succinylacetone, N-acetyl-L-alanine, hippuric acid, 2-ketoglutaric acid and citric acid are organic acids with either a steric hindrance or keto-/amino group(s) that have resulted in the formation of multiple derivatives. The addition of a methoxymation step resulted in one less derivative for 2-ketoglutaric acid. From a reproducibility viewpoint, adapted thermal derivatisation provided better repeatability except for hippuric acid that showed poor repeatability for all types of derivatisation. Within thermal- and adapted thermal derivatisation the lowest temperature (50°C) and shortest reaction time (30 min) showed the least reproducible derivatisation for orotic acid. The results demonstrate that derivatisation efficiency is affected by the chemical structures of the organic acids and also the derivatisation conditions used.

Coefficient of variance was used as the main performance criterion in this study and used as a mean to compare the most repeatable derivatisation condition for a selected group of organic acid derivatives as stipulated in **objective four**. From the results of this study, no apparent optimal derivatisation condition was found for the combined group of organic acids investigated. Considering the repeatability within all conditions for all the organic acids included, the following can be concluded from this study. For thermal derivatisation the condition at 70°C for 90 min has given the lowest CV values. Within adapted thermal derivatisation the condition at 60°C for 30 min resulted in the lowest CV values. Microwave-assisted derivatisation at 150 W for 1.5 min ensured the lowest CV values. Adapted microwave-assisted derivatisation at 350 W for 3.0 min was found to be the condition with the lowest CV values. From all conditions within all derivatisation types 60°C for 30 min was identified as the condition within adapted thermal derivatisation to provide the least variation when all included organic acids are required to be analysed.

5.3 Final remarks

Between conventional thermal- and microwave-assisted derivatisation, conventional thermal derivatisation was found to provide lower variation and to be more robust since less variation was found within conditional changes. As for microwave-assisted derivatisation, it is important to apply the conditions without variation with regards to microwave energy and reaction time. This is generally easier to control with a microwave than in an oven since microwave energy stops with set time. The use of methoxymation was found to be beneficial for repeatability of some organic acids. It is however not necessarily the addition of methylhydroxylamine hydrochloride that improved derivatisation, but it may be the procedure that included an extended time period for the organic acids to dissolve in pyridine at elevated temperature, that could have had an influence on the results. From a time saving perspective, this may be a disadvantage that needs to be taken in account when total duration of sample processing is an issue. Microwave-assistance in contrast can derivatise organic acids in a much shorter time period, but as was seen in this study, higher variation remains a drawback worth future investigation. Microwave-assistance can reduce the reaction time to 1.5 min in comparison with conventional thermal derivatisation in which similar results could be obtained in over 30 min. Due to possible instrument variation, possible uneven heating and volume loss during microwave-assisted- and adapted microwave-assisted derivatisation it is necessary to repeat these two derivatisation types with adjustments to confirm the results. More focus on logistics such as vial-type for adequote sealing, vial position and the filling volume of a vial would be required in future studies. The use of microwave energy to enhance the efficiency of gas chromatography derivatisation has found limited use and acceptance (Soderholm *et al.*, 2010), suggesting more research is needed.

As a final conclusion it can be said that the aim of this investigation to systematically compare the outcome of predefined derivatisation parameters required for thermal- and microwave-assisted

derivatisation as a prerequisite for GC-MS analysis of selected organic acids was successfully achieved.

5.4 Future recommendations

The following recommendations should be viewed as research initiatives directed towards future research aimed at derivatisation for organic acids as a prerequisite for GC-MS analyses.

- Although some investigated organic acids consisted of similar chemical structures, differences were observed in derivatisation efficiency. Further investigation into the effect of parameters on a larger group of compounds that include a larger variety of chemical structures (amongst other amides) would be meaningful especially if they are important for inborn errors of metabolism screening.

- In order to eliminate other factors, analytical standards were used in this study. It will be valuable to repeat this investigation on extracts from biological fluids.

- Other parameters worth investigating, that could improve derivatisation include: reagents used to perform silylation and oxymation, volume of reagent, methoxymation conditions, stability for a longer period, other derivatisation temperatures, reaction times and microwave energies.

- Results from this study indicated that the addition of the methoxymation step improved repeatability. Determining if the improvement was caused by better solubility due to the extended heating period in pyridine or the influence of methylhydroxylamine hydrochloride will be valuable to improve derivatisation in the future.

- An investigation into factors that may improve microwave-assisted derivatisation repeatability is necessary. Other options to consider to prevent sample loss is the use of applicable screw caps vials (Khoomrung *et al.*, 2015) or to use aluminium crimp cap vails (Damm and Kappe, 2009). In order to assure evenly distributed heating between vials the utilisation of a silicon carbide-based microtiter plate/rotor system can be investigated as suggested by Soderholm *et al.* (2010). Another option to investigate is in the use of a microwave vessel containing water to assist with heat distribution (Khoomrung *et al.*, 2015).

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APPENDIX A: EVALUATION OF OUTLIERS

A.1 Determination of outliers

Outliers were determined from the five replicates in order to determine significant non-parametric variation. Figures A.1 - A.60 elaborate on Sections 4.2.1.1, 4.2.2.1, 4.2.3.1 and 4.2.4.1. All replicates were included in the determination of coefficient of variation values (Chapter 4), since no outliers were determined. Thermal conditions followed by adapted thermal, microwave-assisted and adapted microwave-assisted conditions are presented in order of increasing temperature or microwave energy. For each condition a principal component analysis (PCA) scores plot and a Hotelling's T-squared (T^2) distances plot was constructed.

A.1.1 Thermal derivatisation

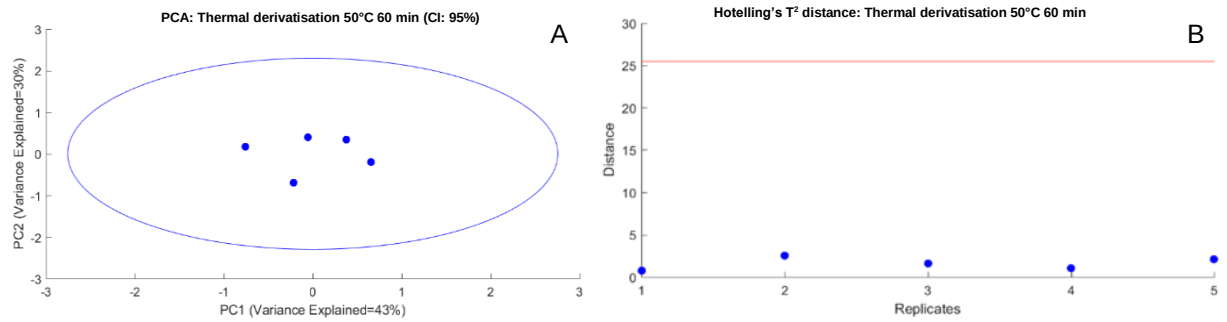

Figure A.1: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of thermal derivatisation at 50°C for 60 min.

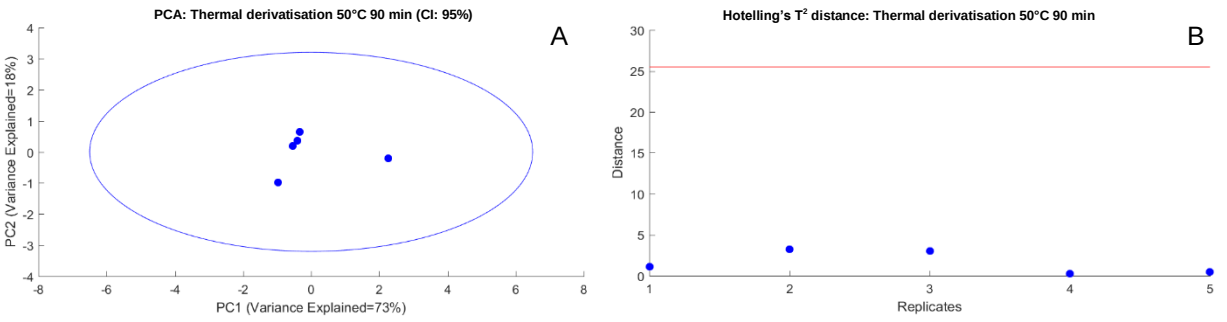

Figure A.2: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of thermal derivatisation at 50°C for 90 min.

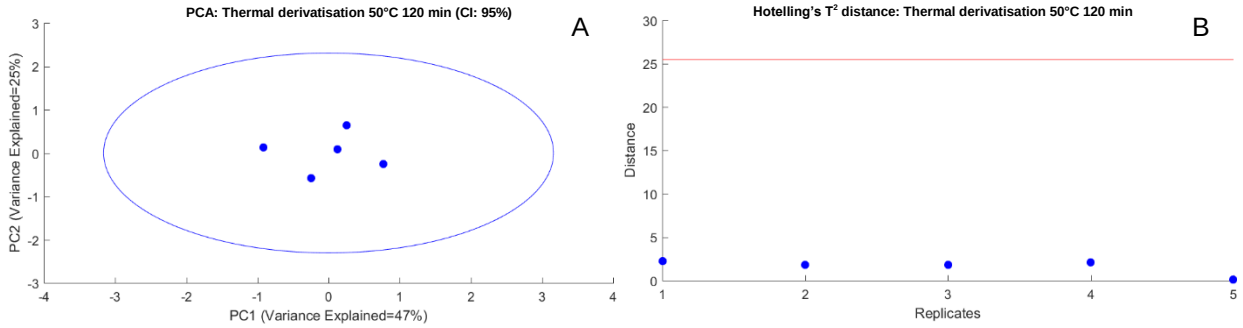

Figure A.3: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of thermal derivatisation at 50°C for 120 min.

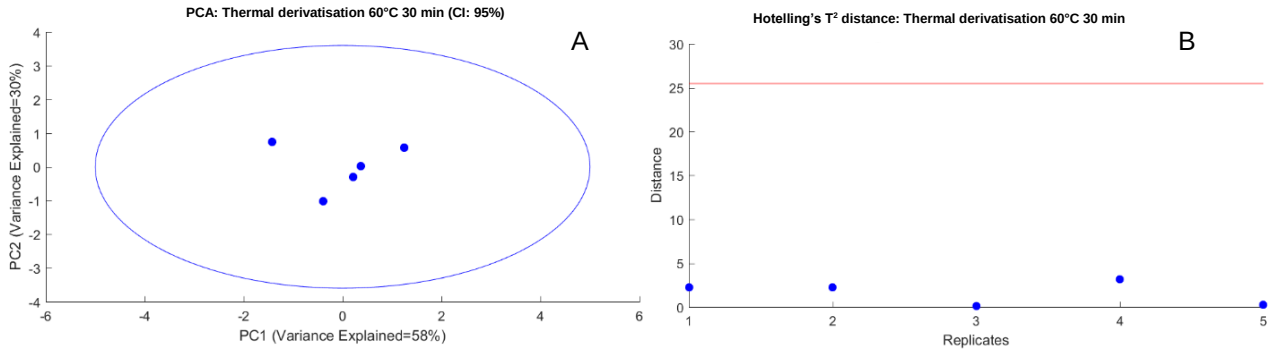

Figure A.4: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of thermal derivatisation at 60°C for 30 min.

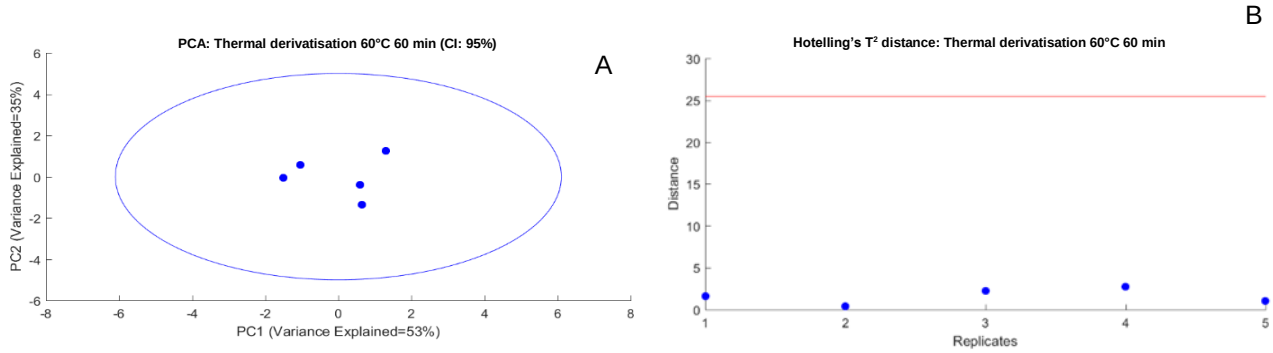

Figure A.5: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of thermal derivatisation at 60°C for 60 min.

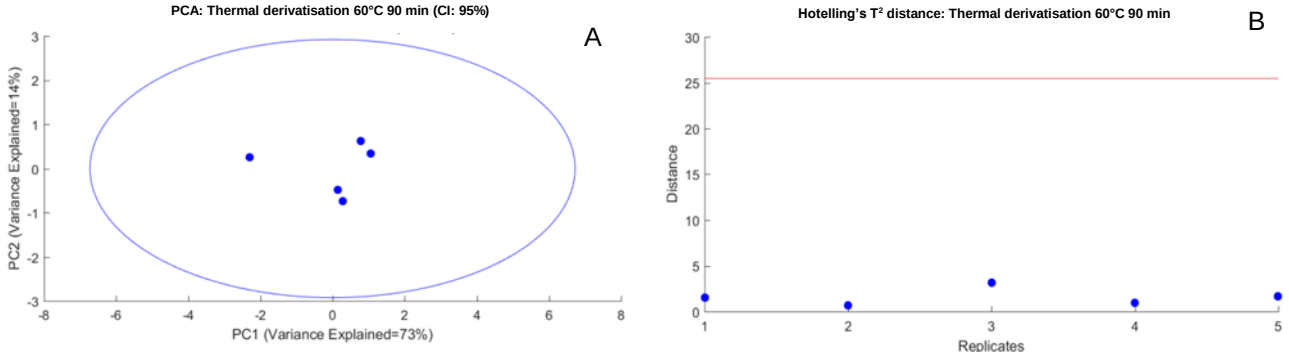

Figure A.6: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of thermal derivatisation at 60°C for 90 min.

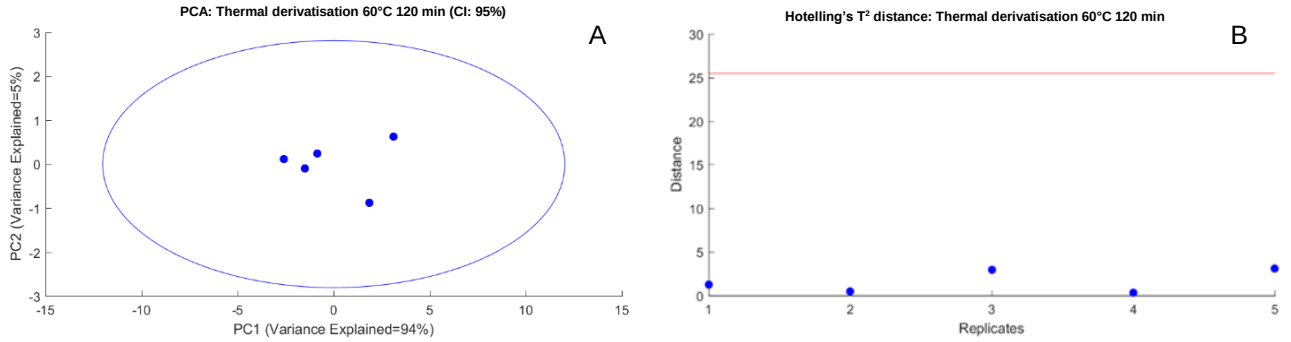

Figure A.7: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of thermal derivatisation at 60°C for 120 min.

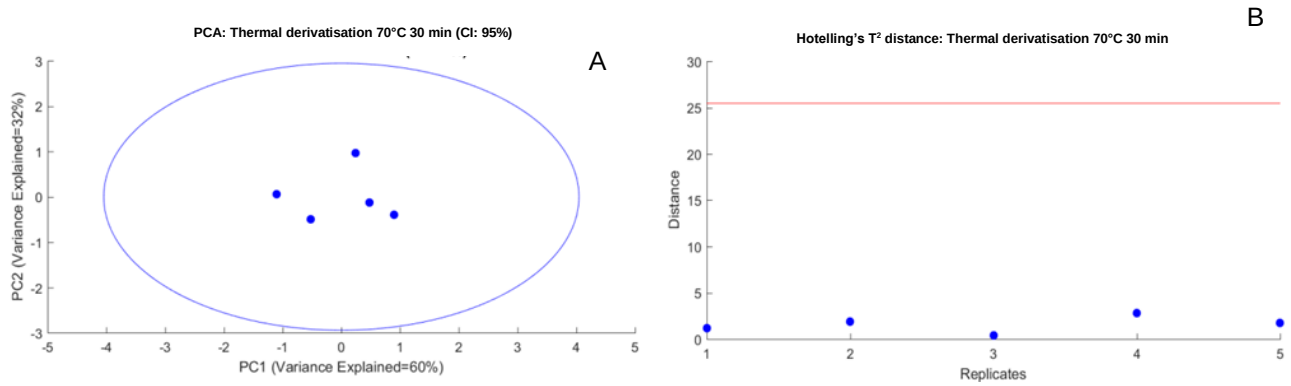

Figure A.8: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of thermal derivatisation at 70°C for 30 min.

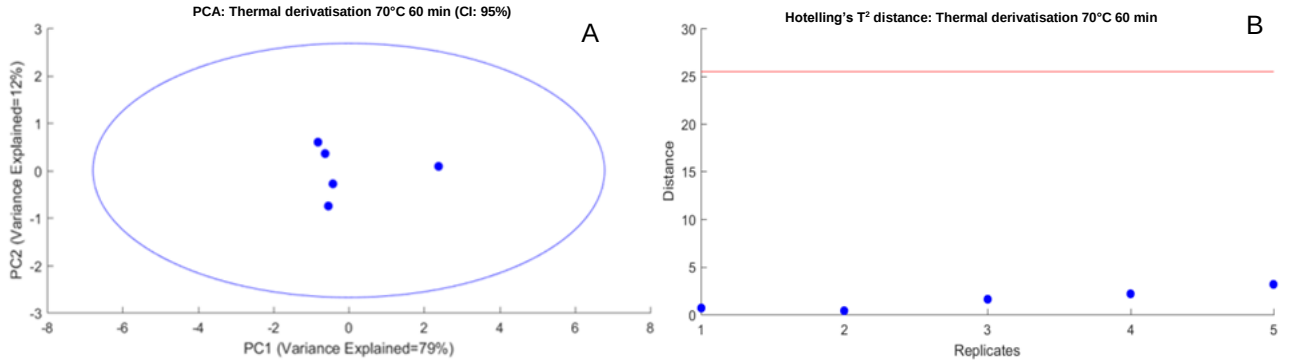

Figure A.9: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of thermal derivatisation at 70°C for 60 min.

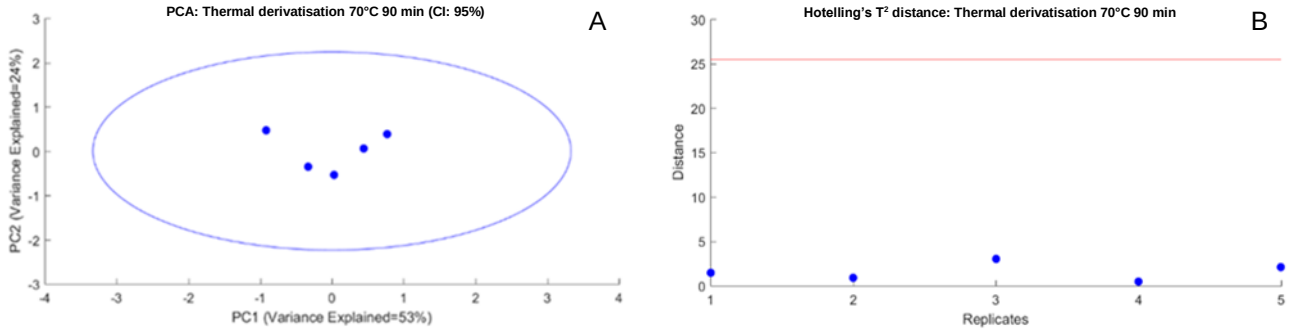

Figure A.10: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of thermal derivatisation at 70°C for 90 min.

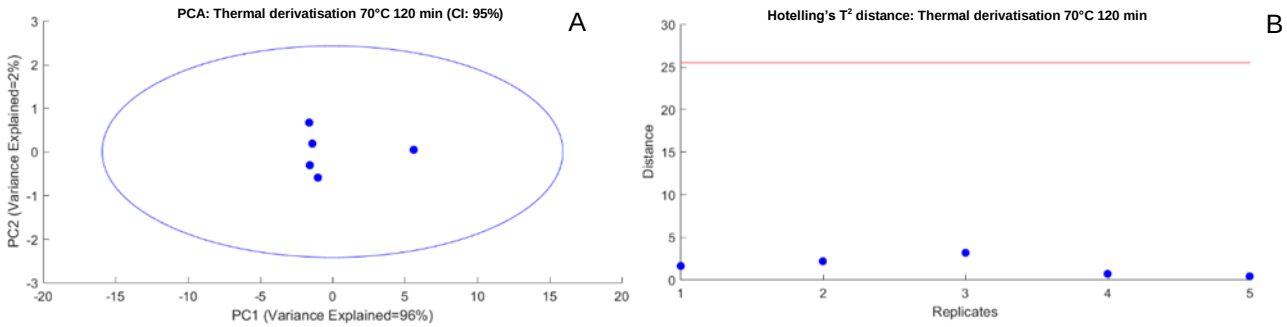

Figure A.11: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of thermal derivatisation at 70°C for 120 min.

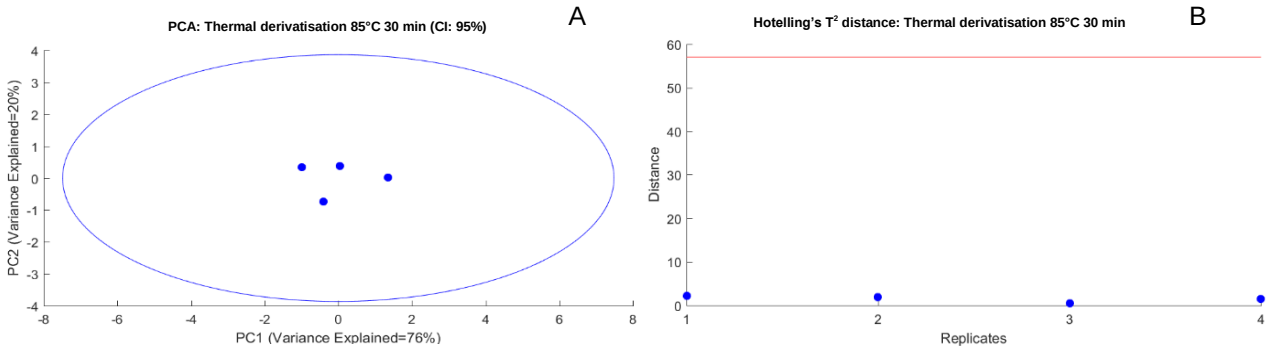

Figure A.12: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of thermal derivatisation at 85°C for 30 min.

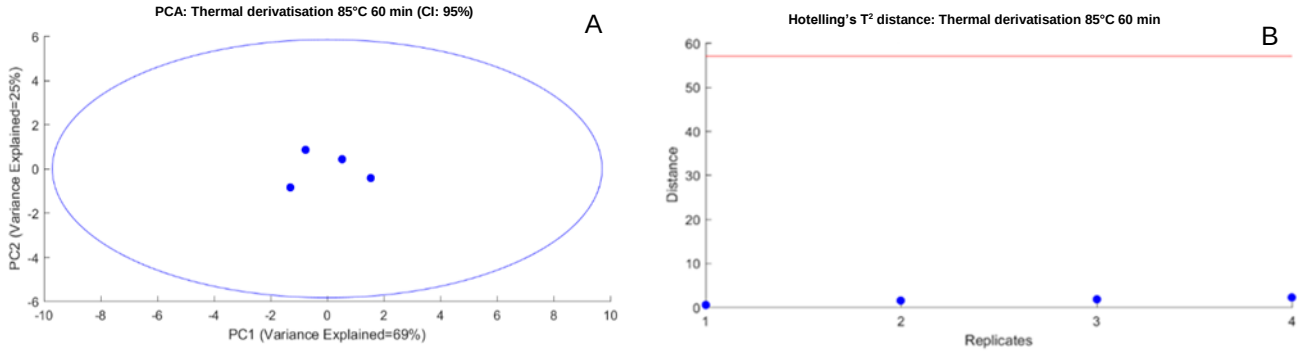

Figure A.13: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of thermal derivatisation at 85°C for 60 min.

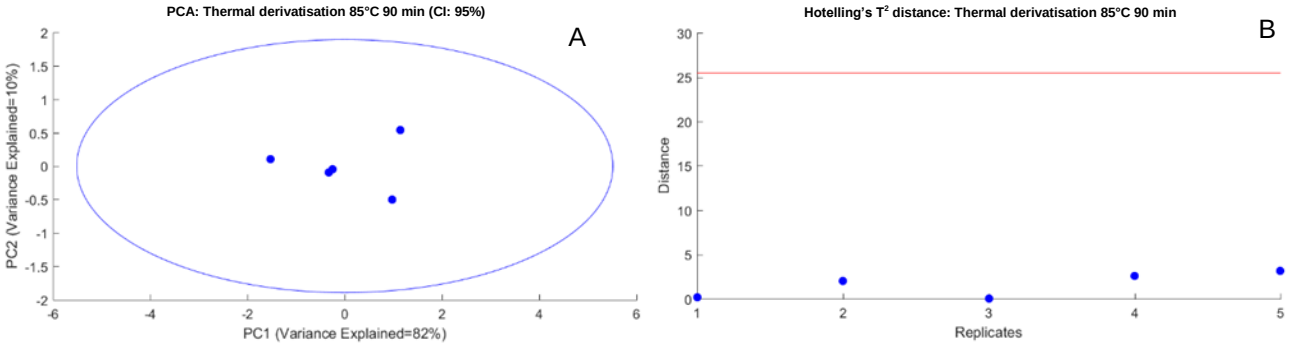

Figure A.14: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of thermal derivatisation at 85°C for 90 min.

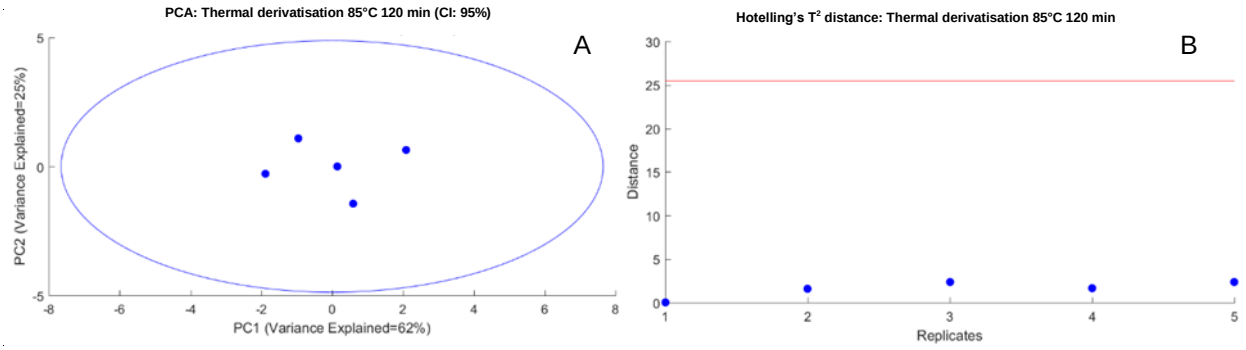

Figure A.15: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of thermal derivatisation at 85°C for 120 min.

A.1.2 Adapted thermal derivatisation

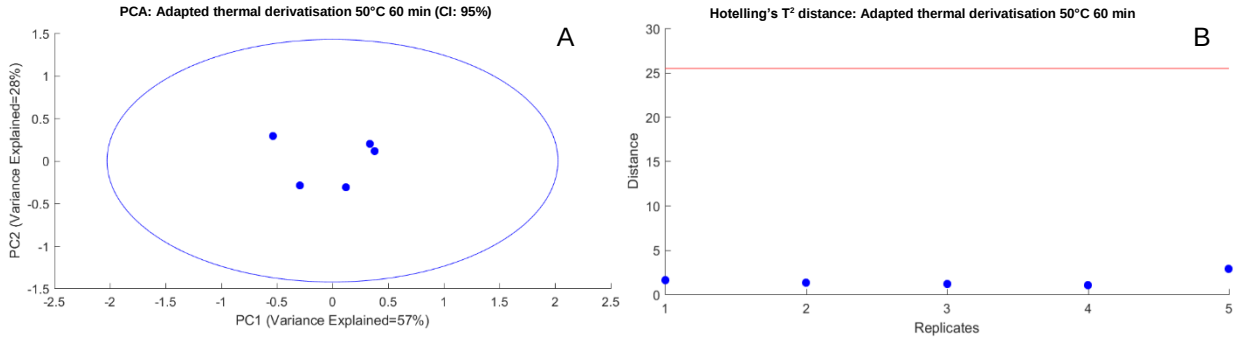

Figure A.16: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted thermal derivatisation at 50°C for 60 min.

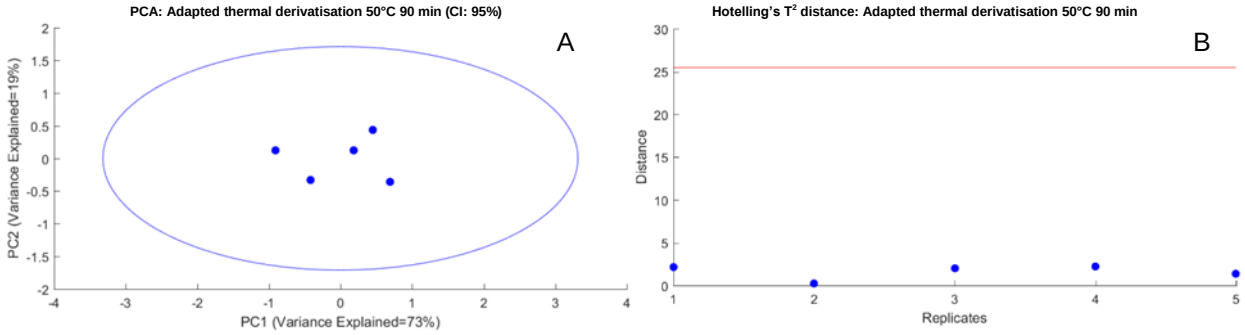

Figure A.17: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted thermal derivatisation at 50°C for 90 min.

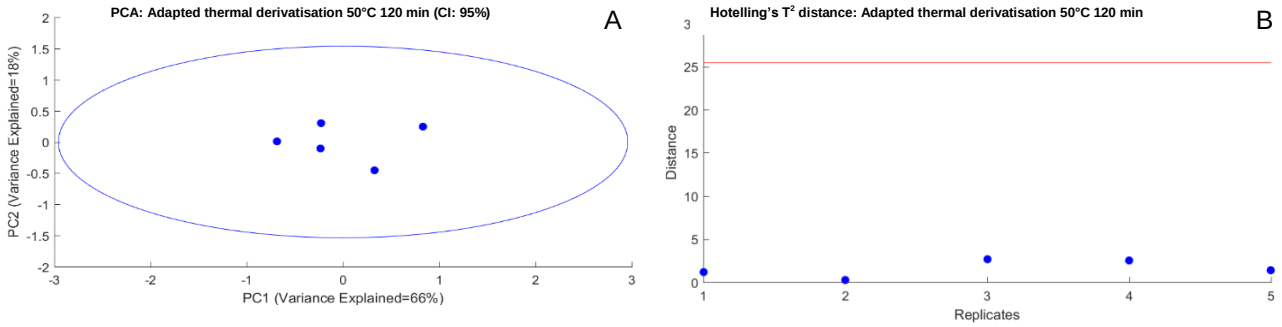

Figure A.18: Outlier evaluation. Principal component analysis (A) and Hotelling's T2 (B) of adapted thermal derivatisation at 50°C for 120 min.

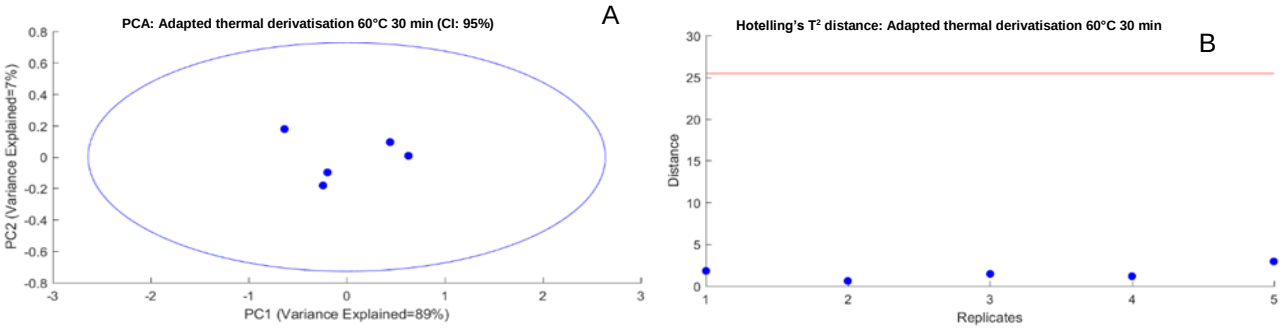

Figure A.19: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted thermal derivatisation at 60°C for 30 min.

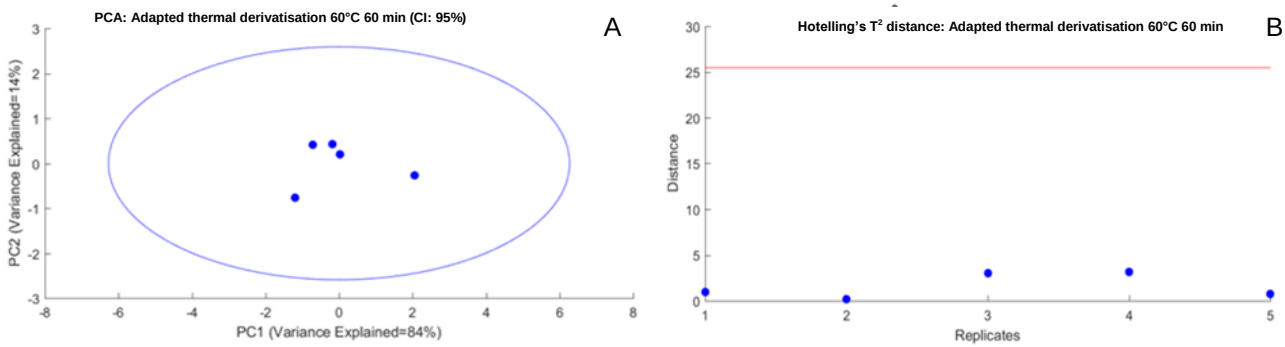

Figure A.20: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted thermal derivatisation at 60°C for 60 min.

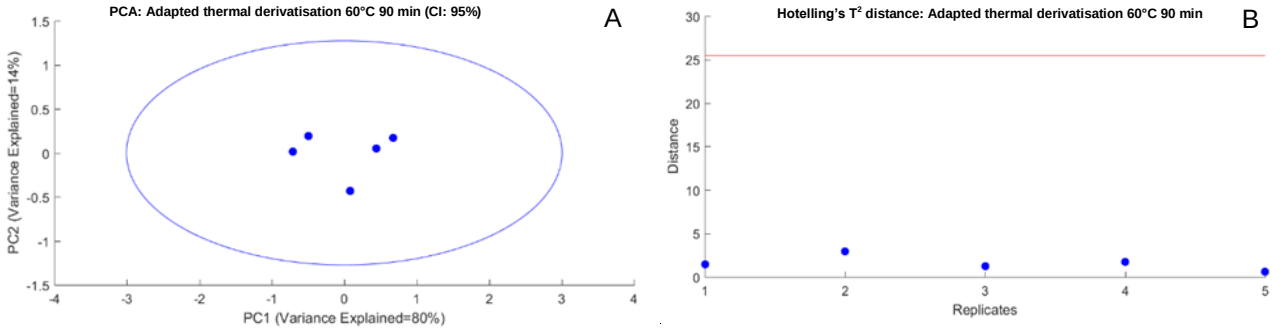

Figure A.21: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted thermal derivatisation at 60°C for 90 min.

Hotelling's T^2 distance: Adapted thermal derivatisation 60°C 120 min B

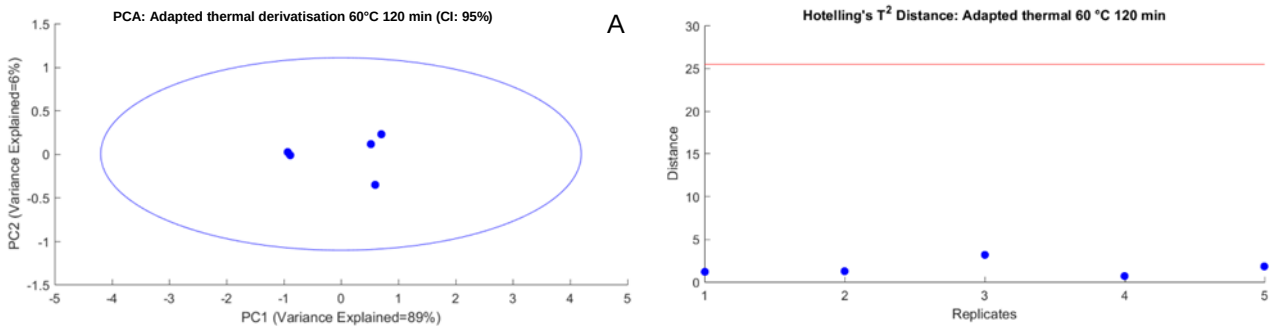

Figure A.22: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted thermal derivatisation at 60°C for 120 min.

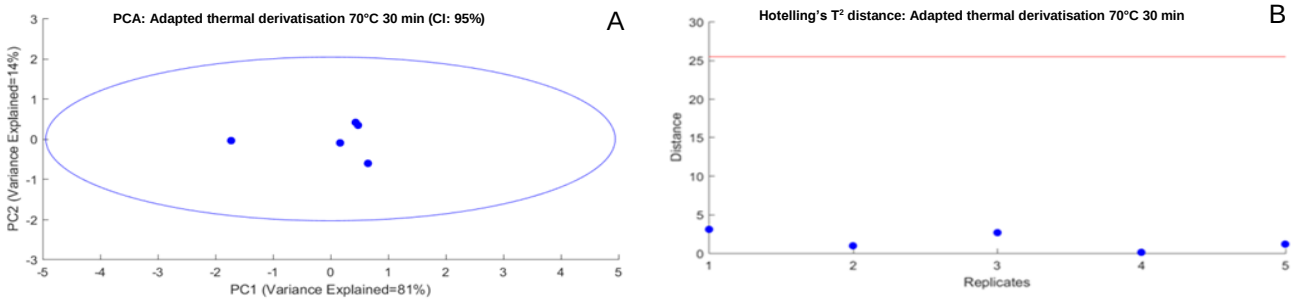

Figure A.23: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted thermal derivatisation at 70°C for 30 min.

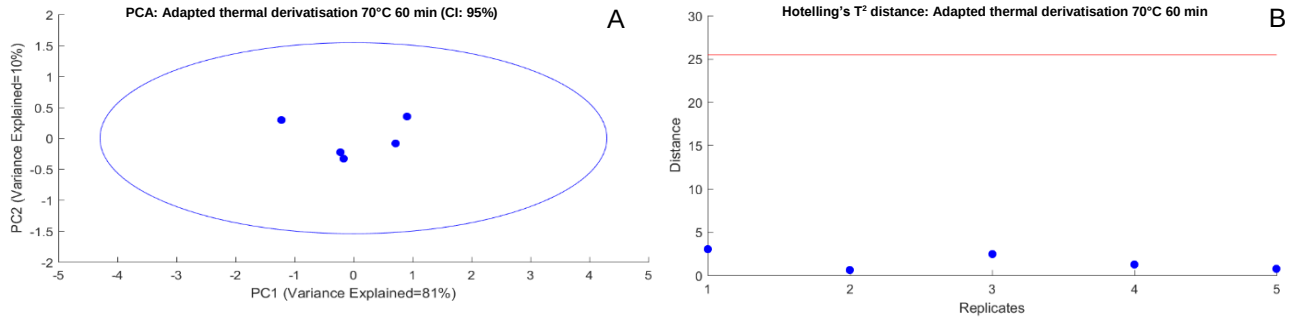

Figure A.24: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted thermal derivatisation at 70°C for 60 min.

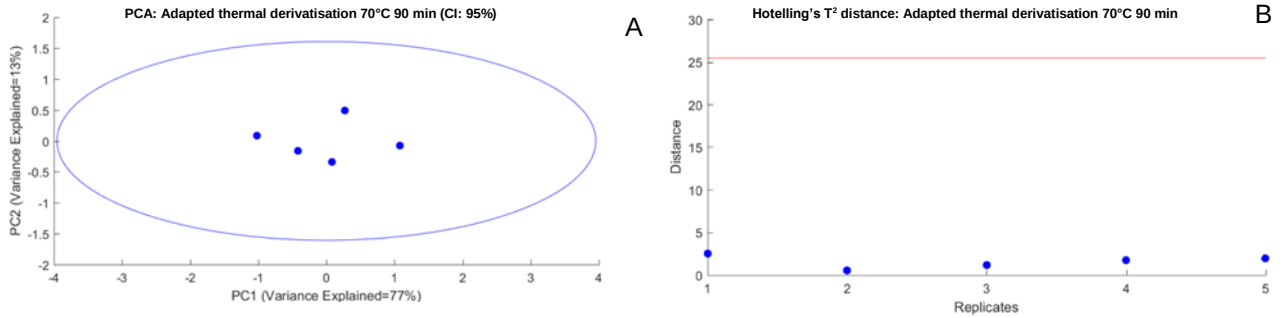

Figure A.25: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted thermal derivatisation at 70°C for 90 min.

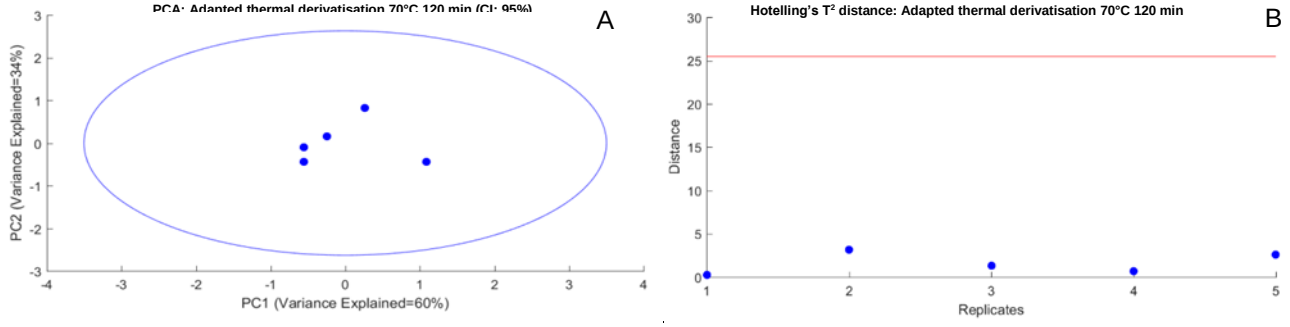

Figure A.26: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted thermal derivatisation at 70°C for 120 min.

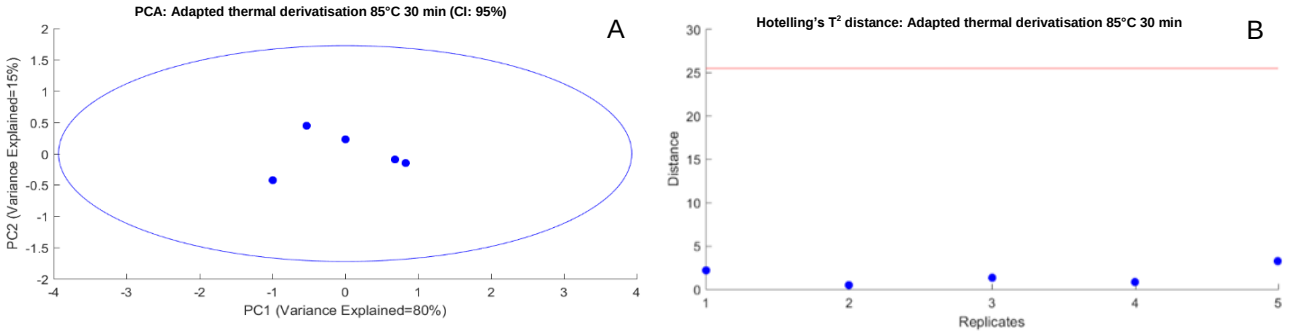

Figure A.27: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted thermal derivatisation at 85°C for 30 min.

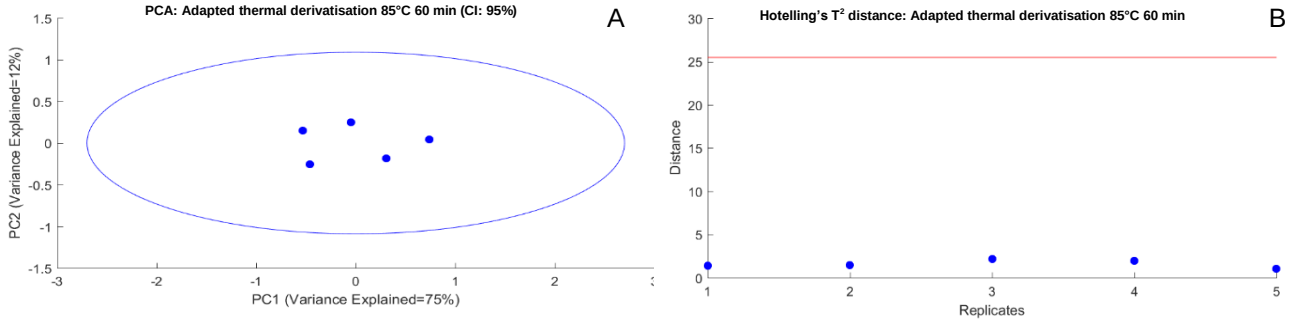

Figure A.28: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted thermal derivatisation at 85°C for 60 min.

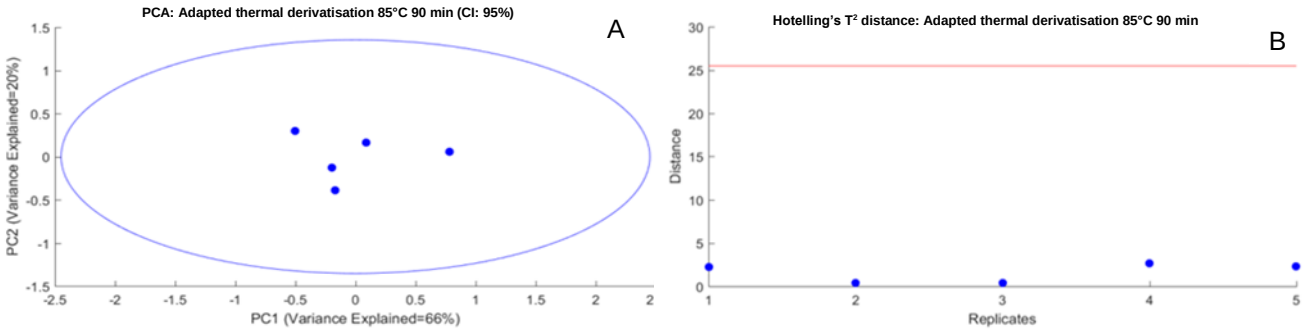

Figure A.29: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted thermal derivatisation at 85°C for 90 min.

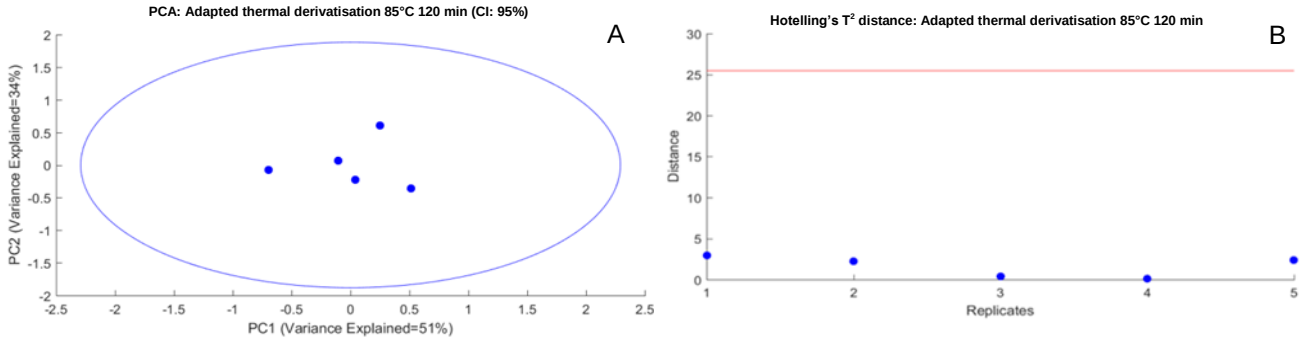

Figure A.30: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted thermal derivatisation at 85°C for 120 min.

A.1.3 Microwave-assisted derivatisation

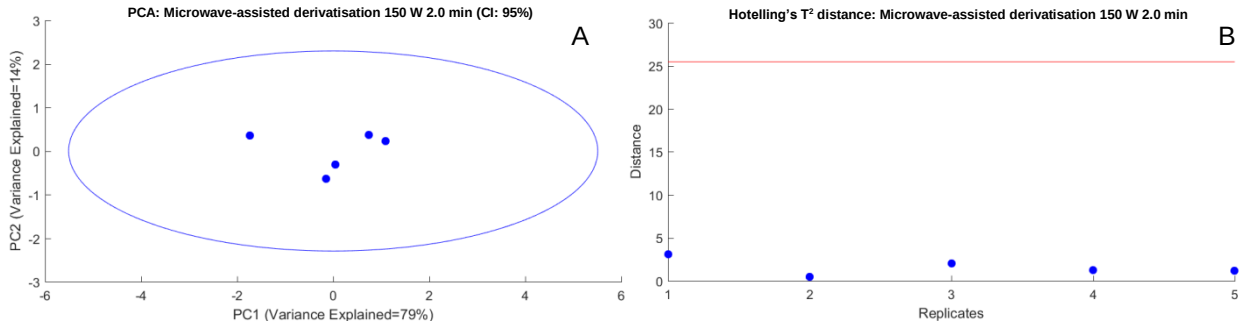

Figure A.31: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of microwave-assisted derivatisation at 150 W for 2.0 min.

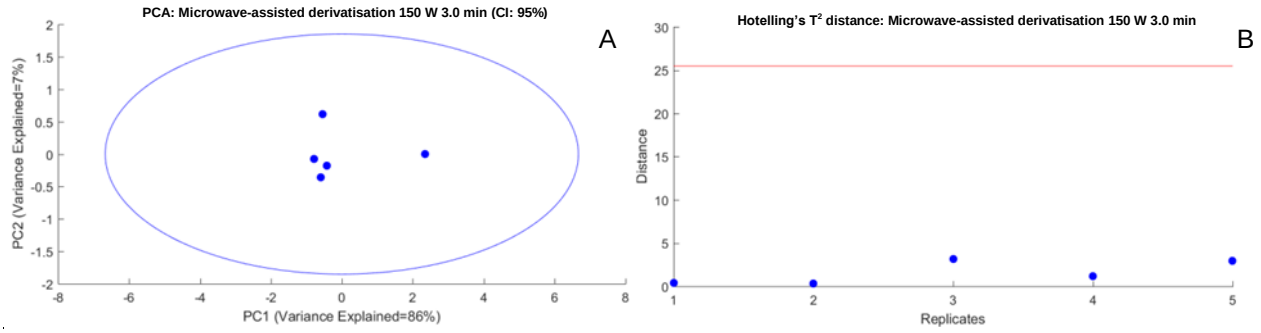

Figure A.32: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of microwave-assisted derivatisation at 150 W for 3.0 min.

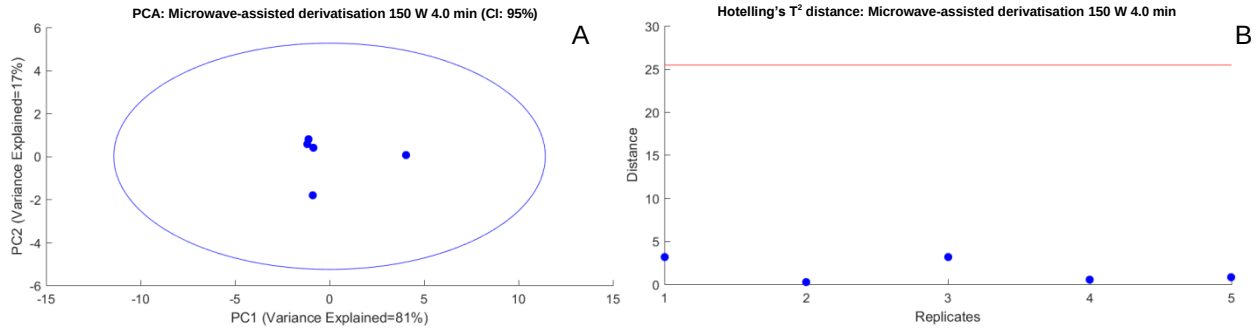

Figure A.33: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of microwave-assisted derivatisation at 150 W for 4.0 min.

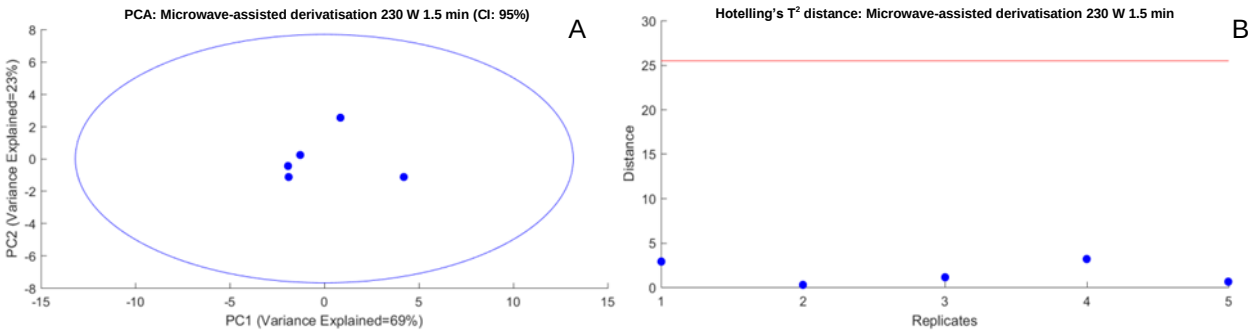

Figure A.34: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of microwave-assisted derivatisation at 230 W for 1.5 min.

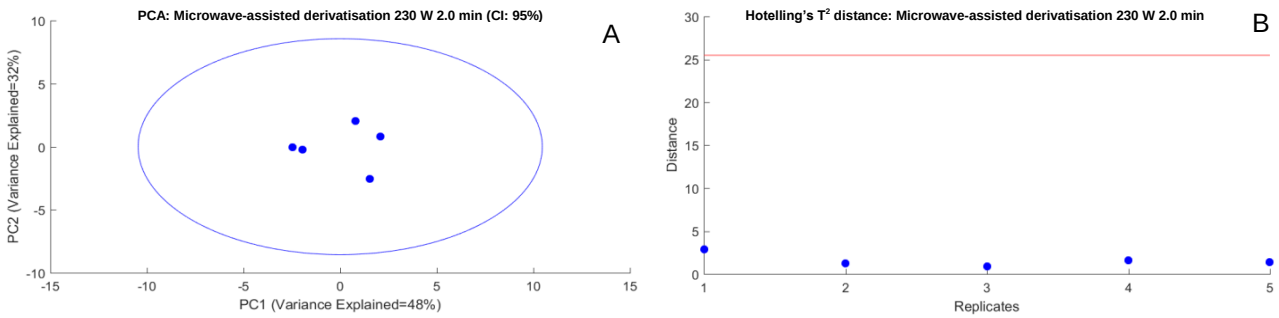

Figure A.35: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of microwave-assisted derivatisation at 230 W for 2.0 min.

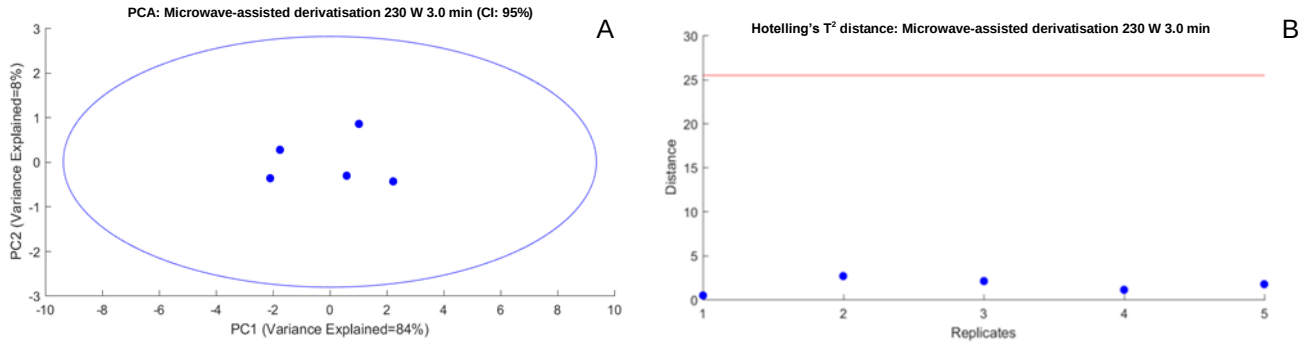

Figure A.36: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of microwave-assisted derivatisation at 230 W for 3.0 min.

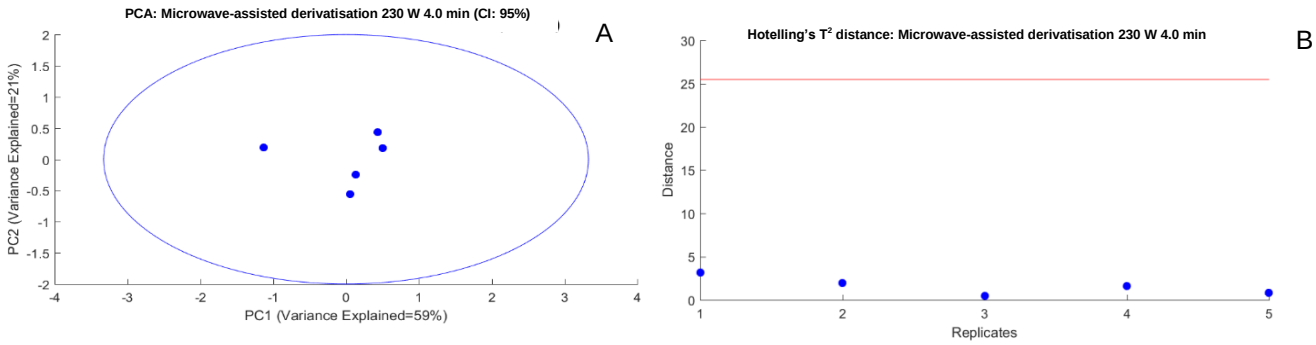

Figure A.37: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of microwave-assisted derivatisation at 230 W for 4.0 min.

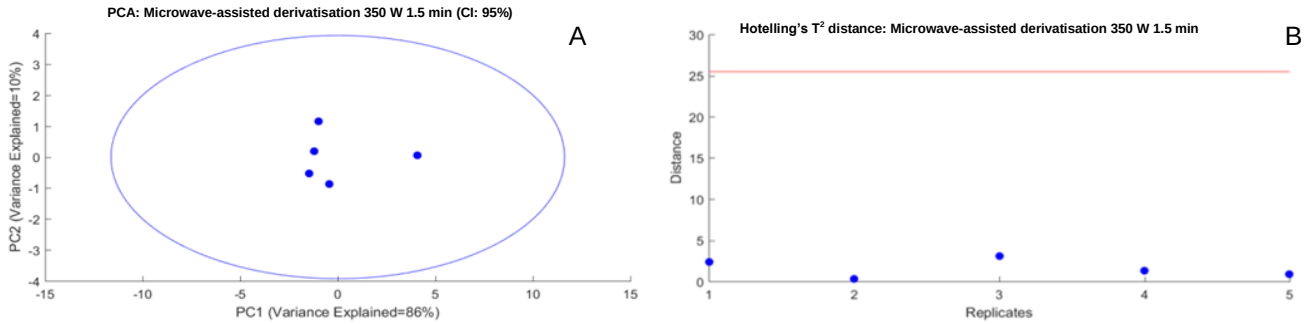

Figure A.38: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of microwave-assisted derivatisation at 350 W for 1.5 min.

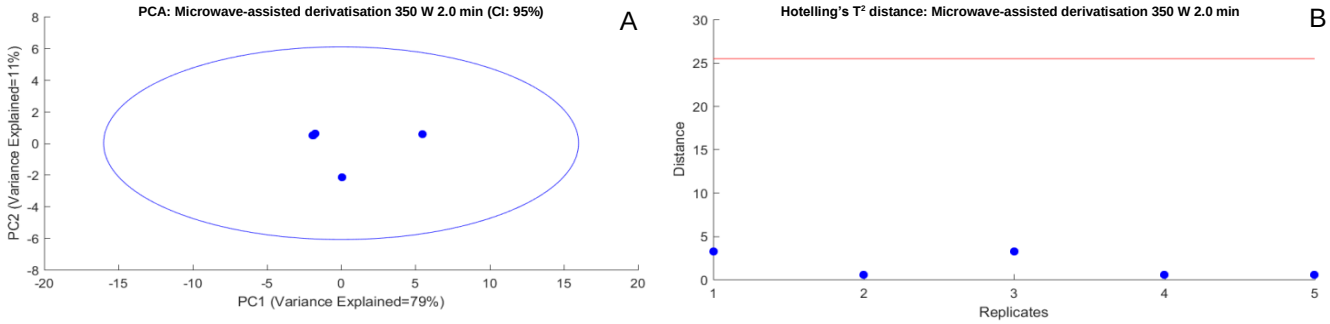

Figure A.39: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of microwave-assisted derivatisation at 350 W for 2.0 min.

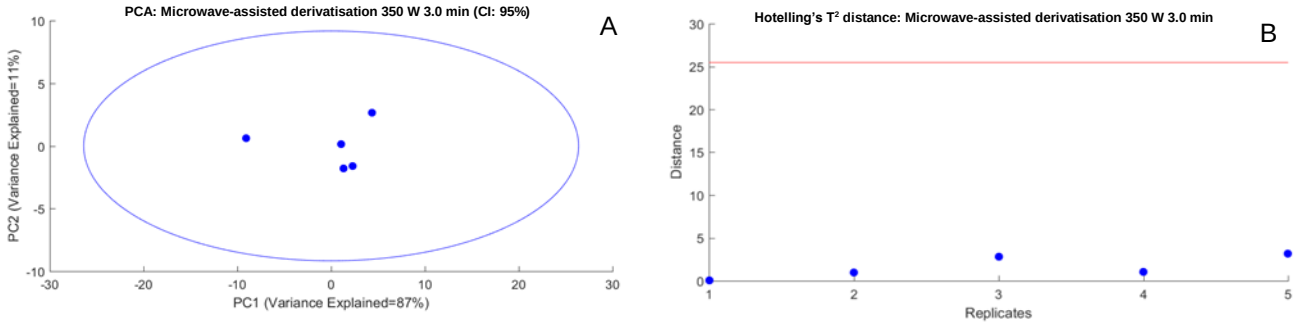

Figure A.40: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of microwave-assisted derivatisation at 350 W for 3.0 min.

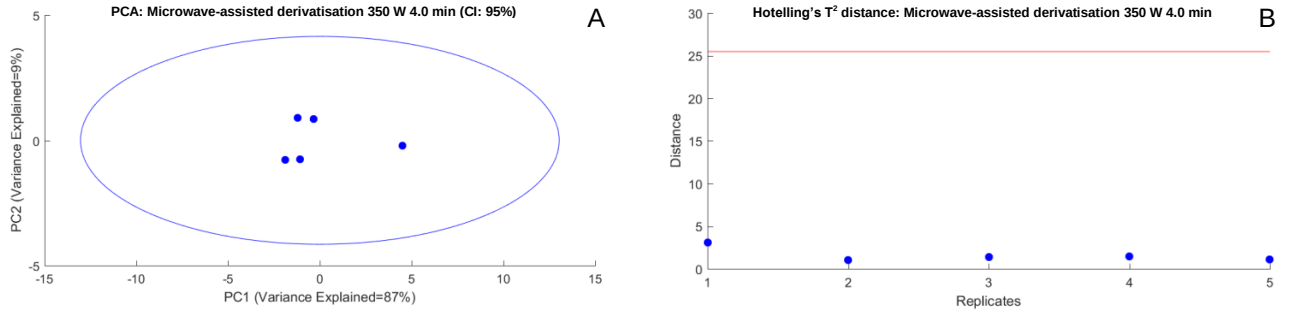

Figure A.41: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of microwave-assisted derivatisation at 350 W for 4.0 min.

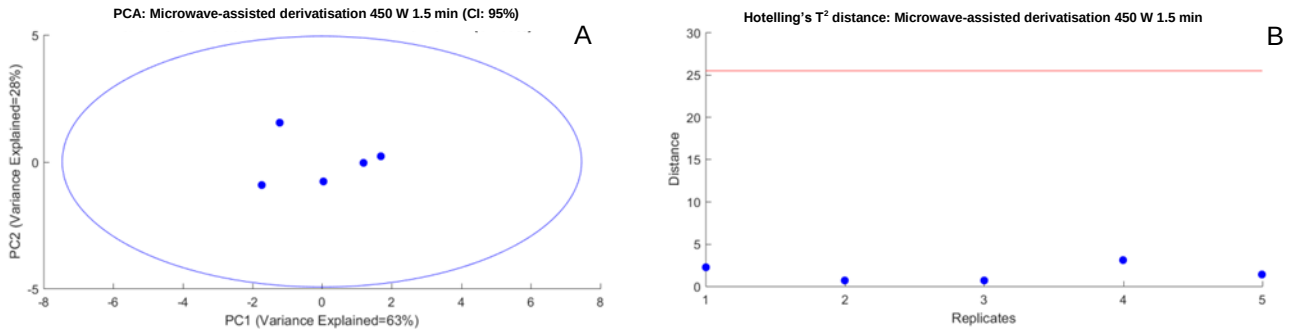

Figure A.42: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of microwave-assisted derivatisation at 450 W for 1.5 min.

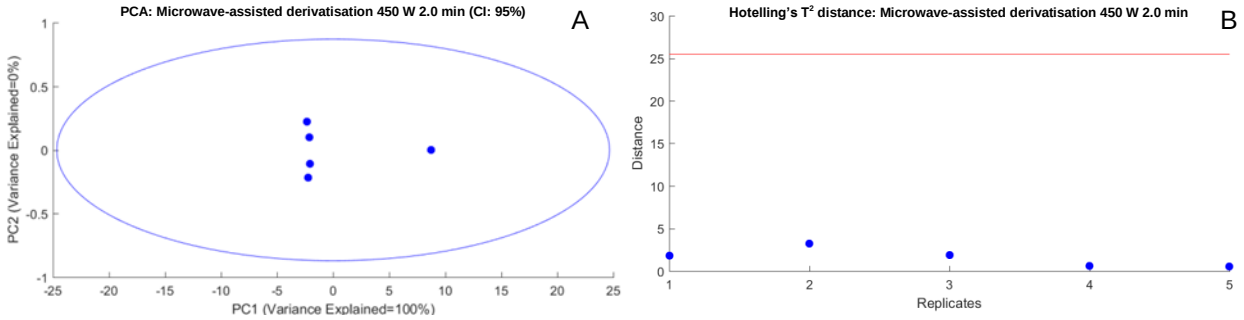

Figure A.43: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of microwave-assisted derivatisation at 450 W for 2.0 min.

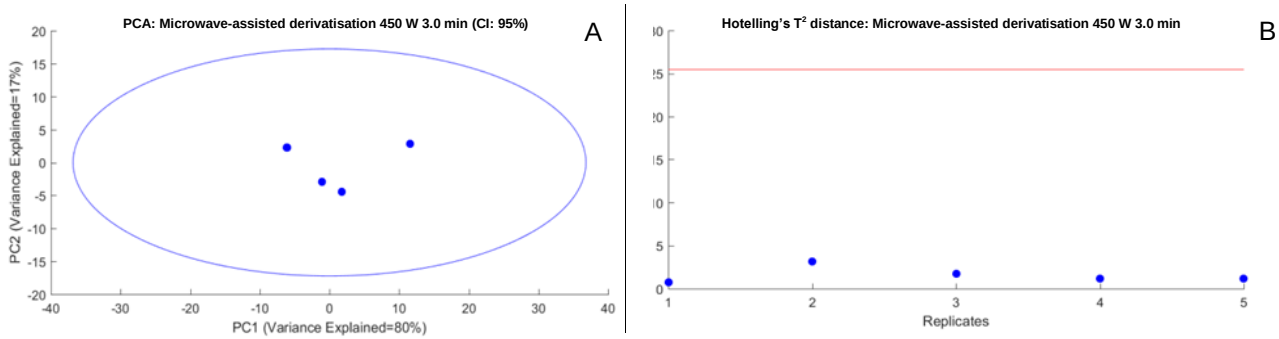

Figure A.44: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of microwave-assisted derivatisation at 450 W for 3.0 min.

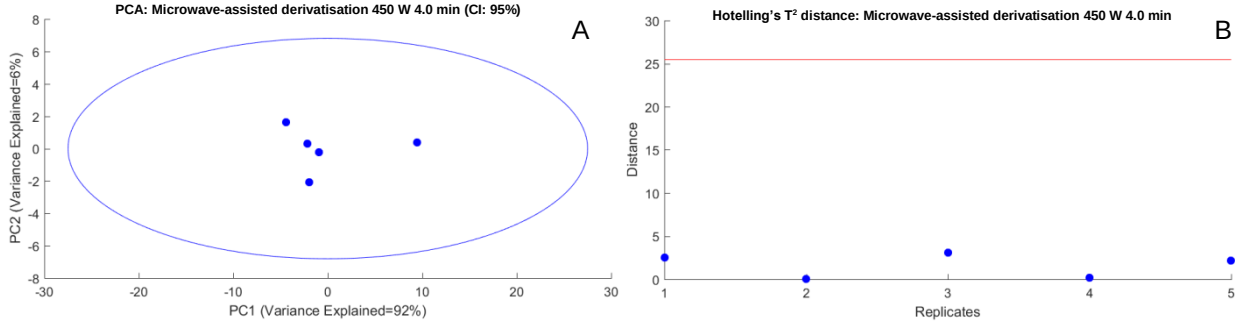

Figure A.45: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of microwave-assisted derivatisation at 450 W for 4.0 min.

A.1.4 Adapted microwave-assisted derivatisation

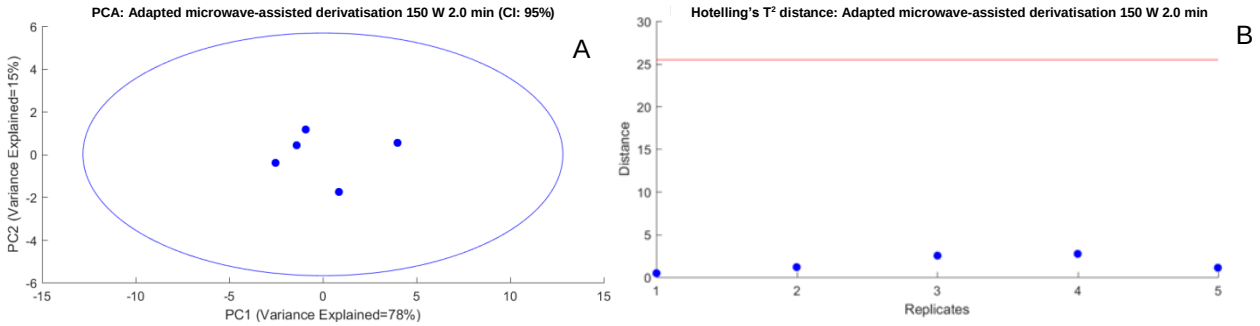

Figure A.46: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted microwave-assisted derivatisation at 150 W for 2.0 min.

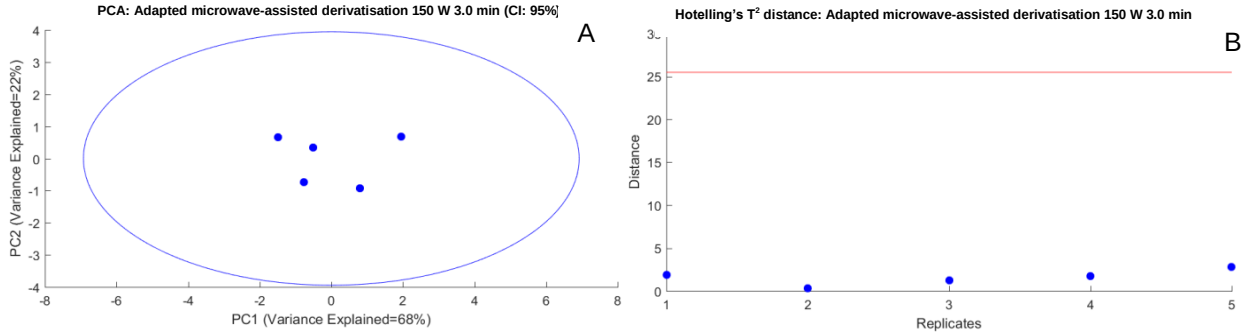

Figure A.47: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted microwave-assisted derivatisation at 150 W for 3.0 min.

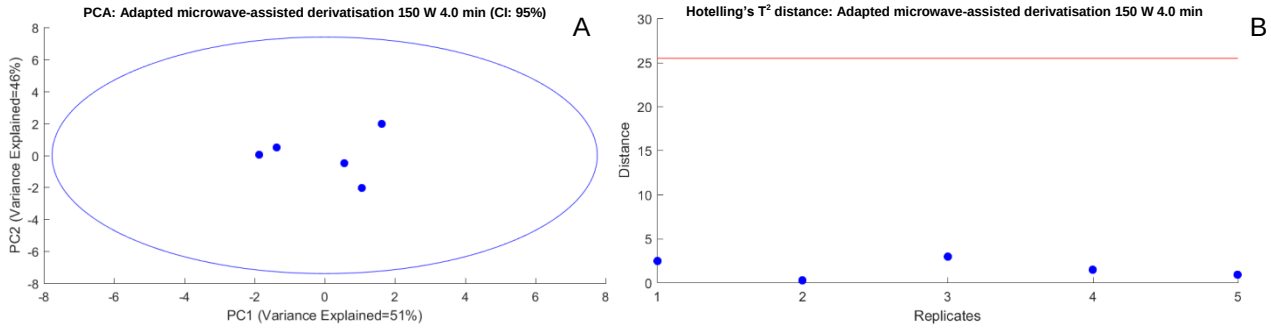

Figure A.48: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted microwave-assisted derivatisation at 150 W for 4.0 min.

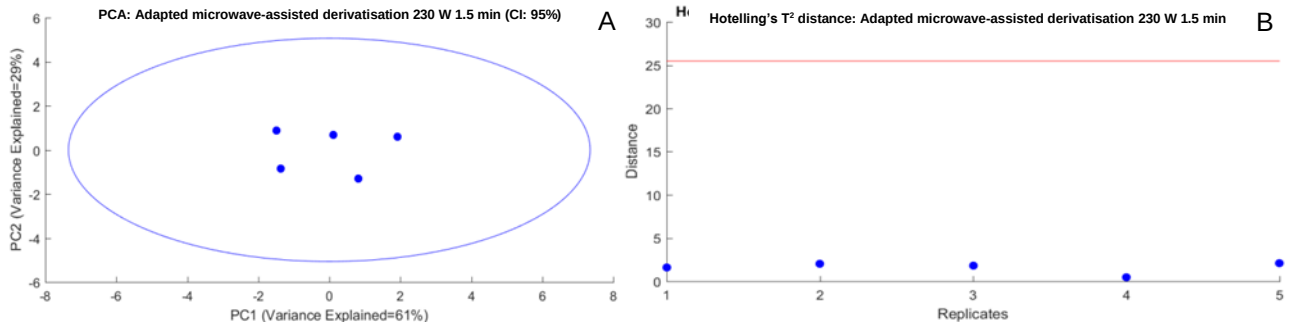

Figure A.49: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted microwave-assisted derivatisation at 230 W for 1.5 min.

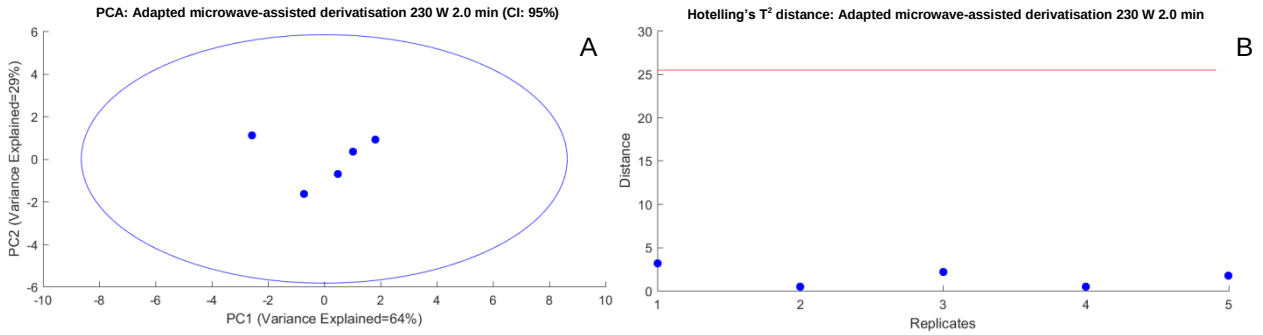

Figure A.50: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted microwave-assisted derivatisation at 230 W for 2.0 min.

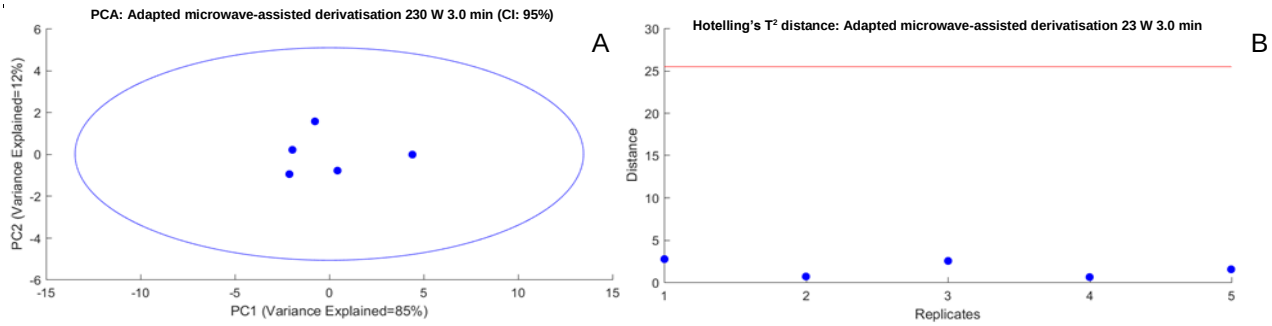

Figure A.51: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted microwave-assisted derivatisation at 230 W for 3.0 min.

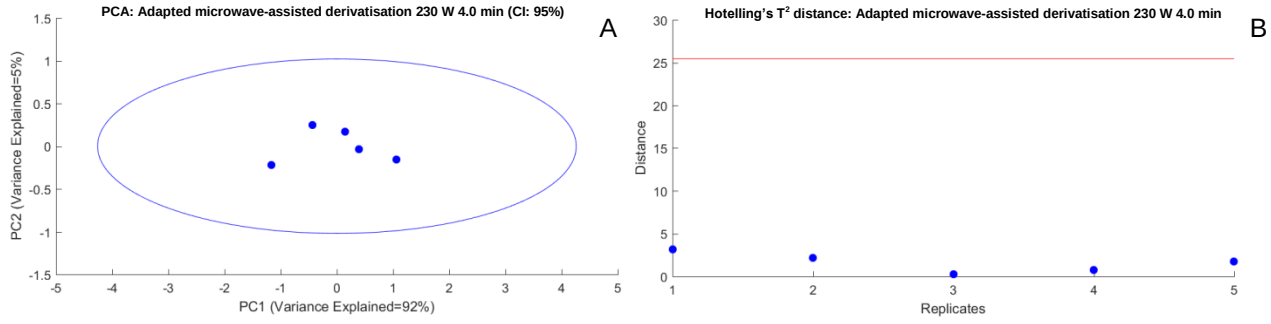

Figure A.52: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted microwave-assisted derivatisation at 230 W for 4.0 min.

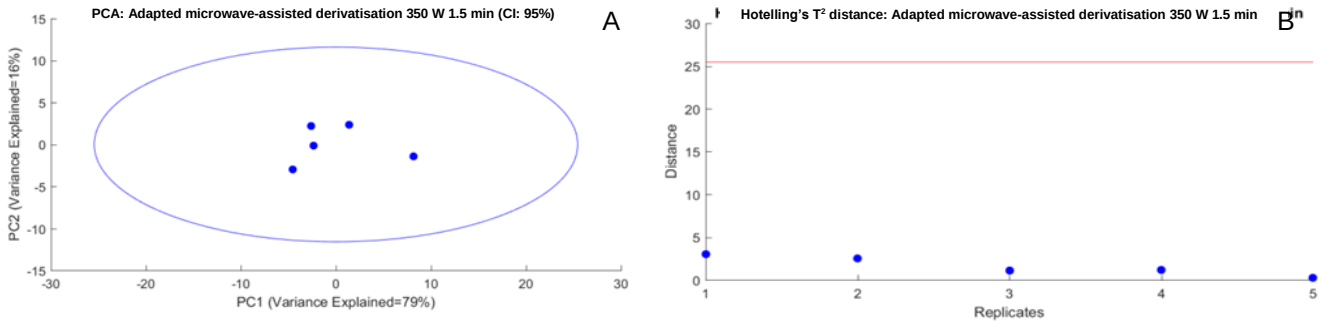

Figure A.53: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted microwave-assisted derivatisation at 350 W for 1.5 min.

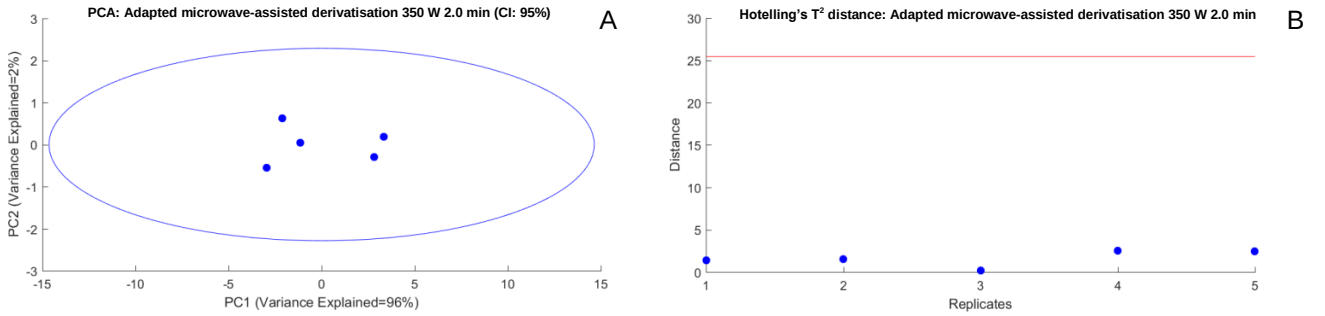

Figure A.54: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted microwave-assisted derivatisation at 350 W for 2.0 min.

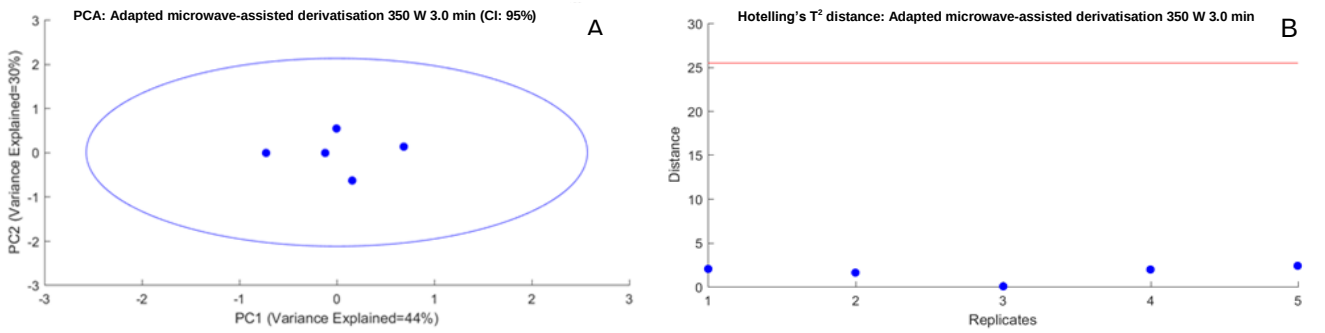

Figure A.55: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted microwave-assisted derivatisation at 350 W for 3.0 min.

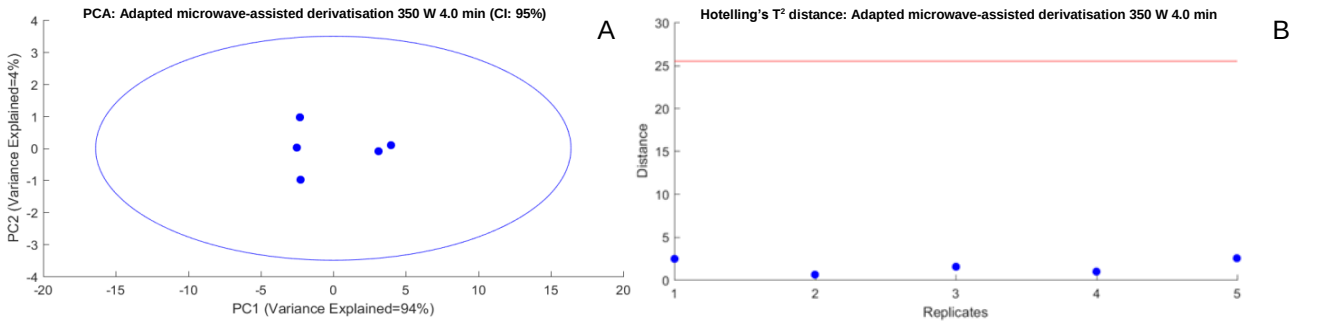

Figure A.56: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted microwave-assisted derivatisation at 350 W for 4.0 min.

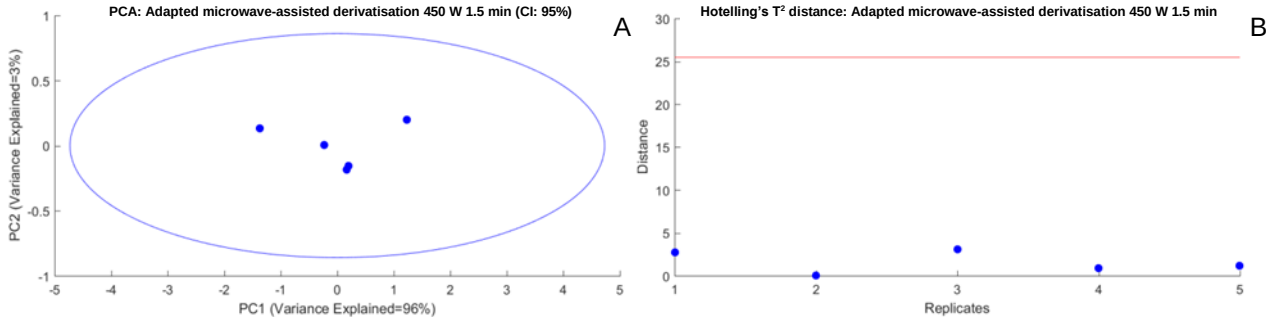

Figure A.57: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted microwave-assisted derivatisation at 450 W for 1.5 min.

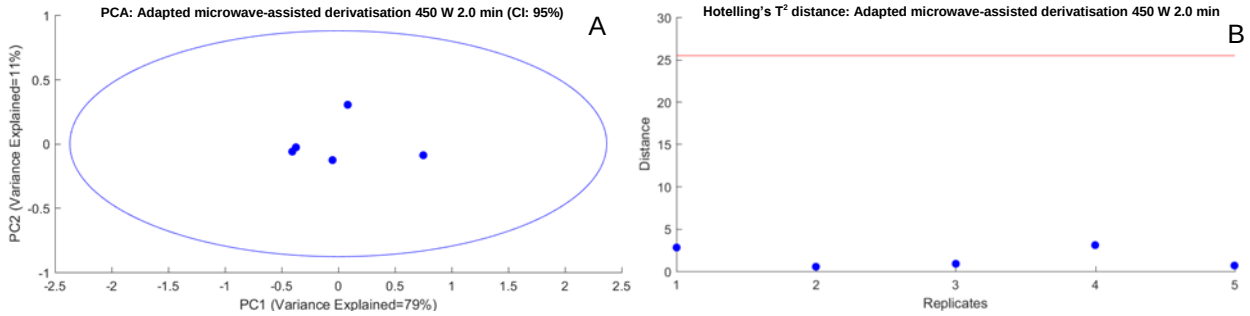

Figure A.58: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted microwave-assisted derivatisation at 450 W for 2.0 min.

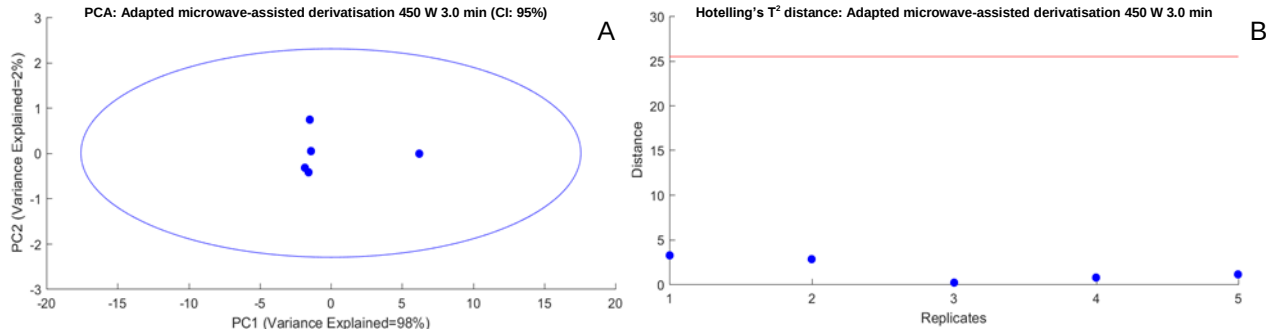

Figure A.59: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted microwave-assisted derivatisation at 450 W for 3.0 min.

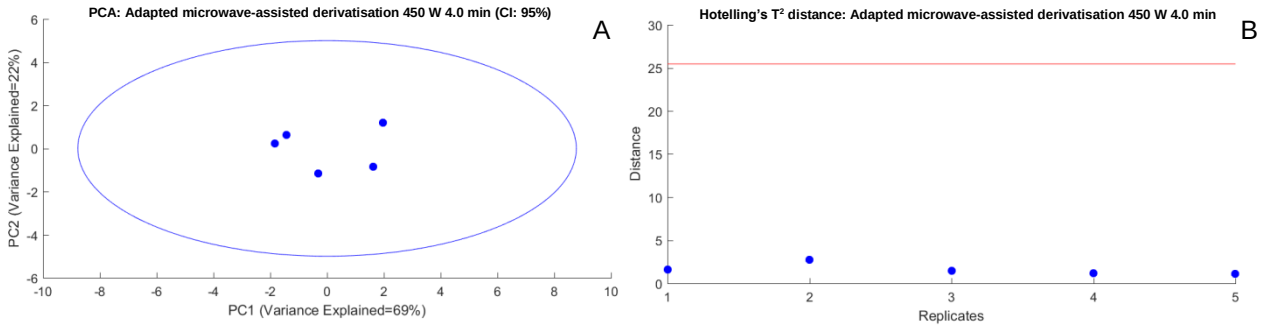

Figure A.60: Outlier evaluation. Principal component analysis (A) and Hotelling's T^2 (B) of adapted microwave-assisted derivatisation at 450 W for 4.0 min.

APPENDIX B: Sample comparison

B.1 Comparison within derivatisation types

Cumulative distribution plots were constructed in order to compare coefficient of variance (CV) of all included organic acids at all conditions within a derivatisation type (Figs. B.1 - B.4). From these cumulative plots the condition within thermal-, adapted thermal-, microwave-assisted- and adapted microwave-assisted derivatisation, with the lowest CV value, was identified and plotted togehter to construct Figure 4.21 in Chapter 4.

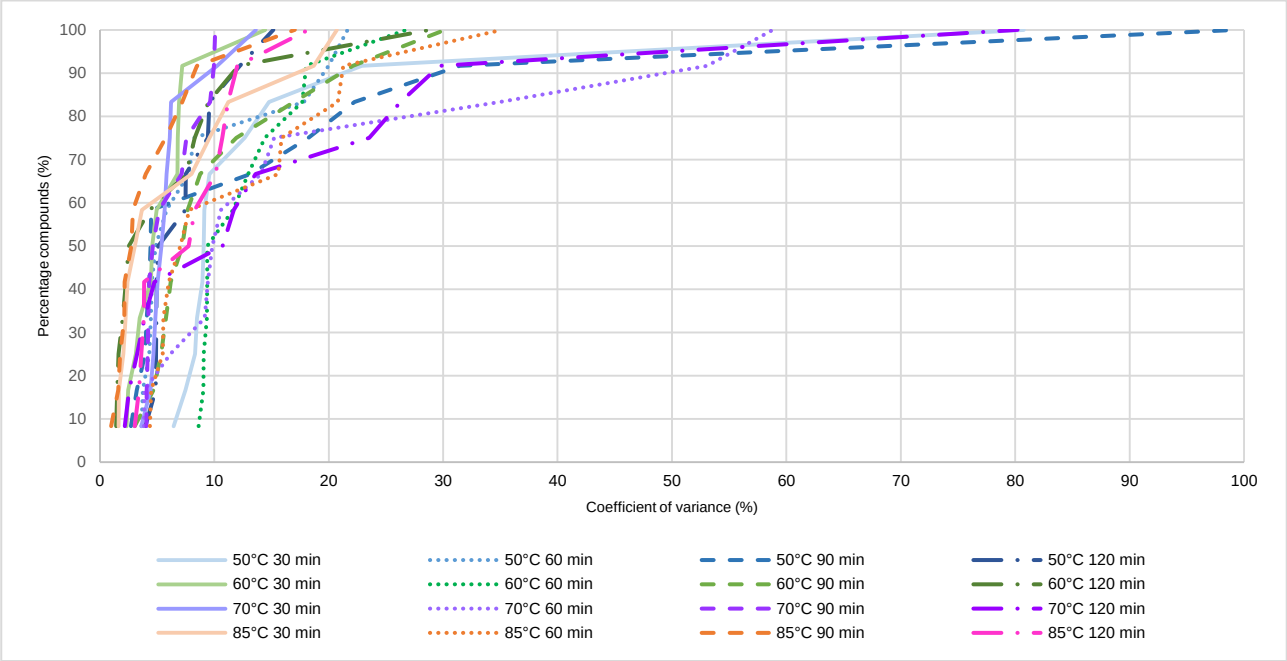

Figure B.1: Cumulative distribution plot of thermal derivatisation conditions. Amount of organic acids (%) and the CV (%) obtained for the organic acids after derivatisation and GC-MS analysis.

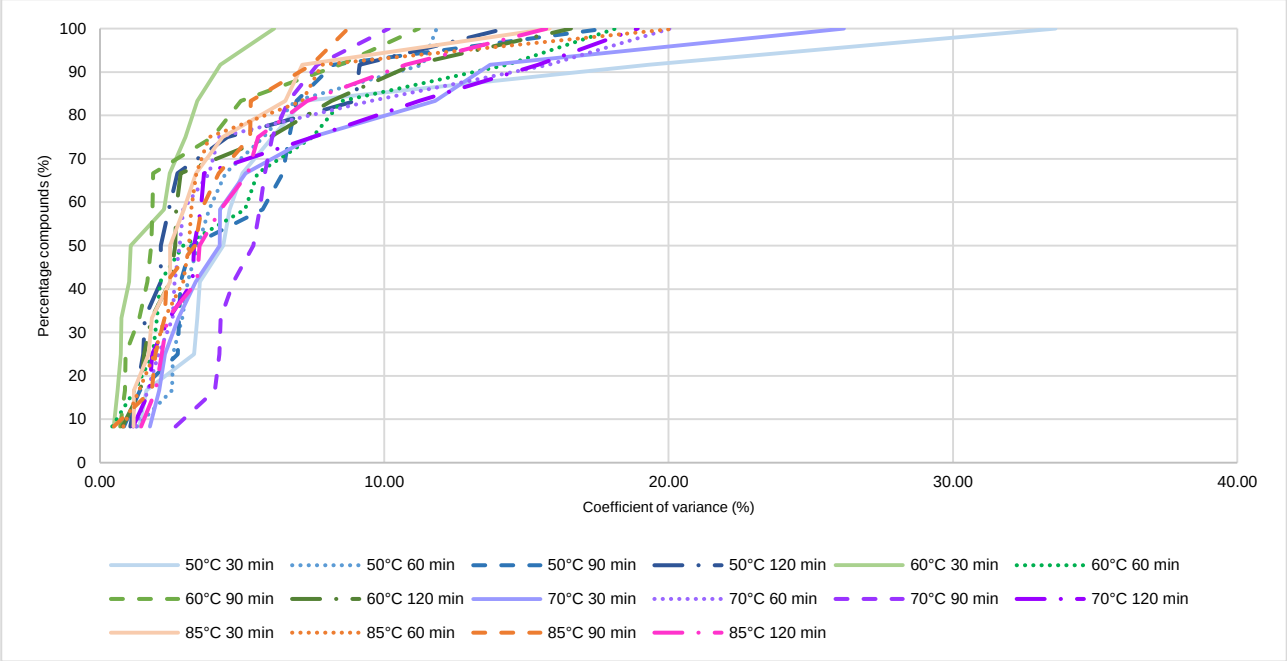

Figure B.2: Cumulative distribution plot of adapted thermal derivatisation conditions. Amount of organic acids (%) and the CV (%) obtained for the organic acids after derivatisation and GC-MS analysis.

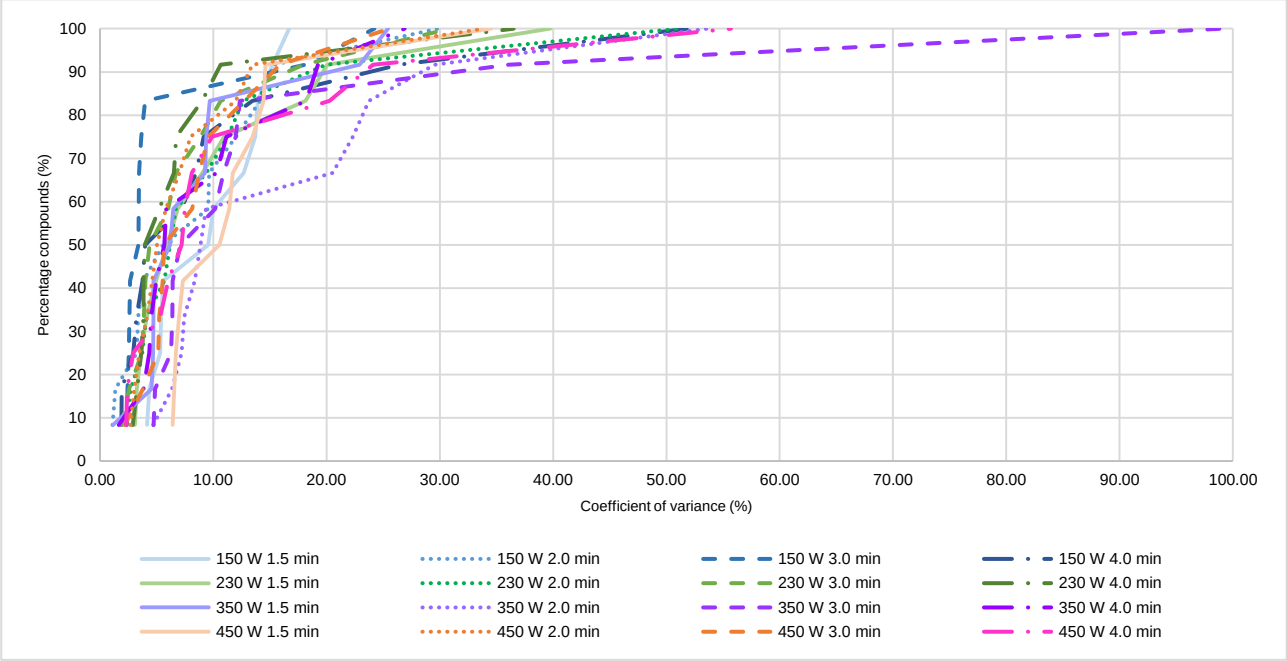

Figure B.3: Cumulative distribution plot of microwave-assisted derivatisation conditions. Amount of organic acids (%) and the CV (%) obtained for the organic acids after derivatisation and GC-MS analysis.

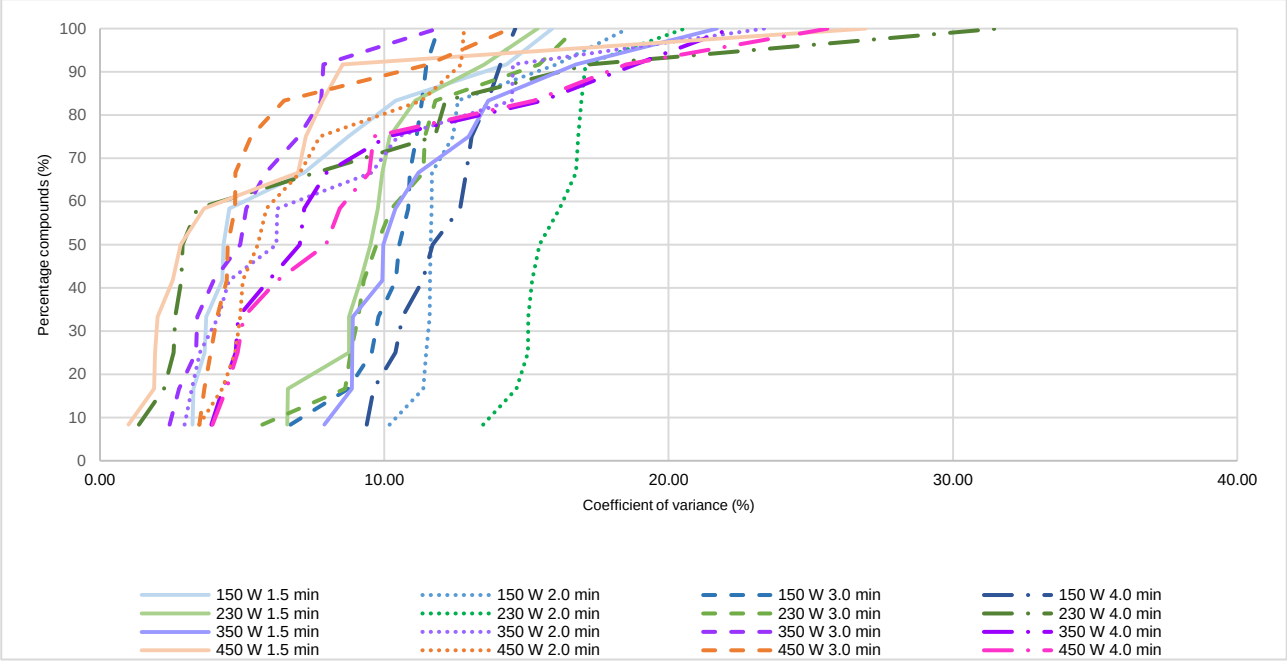

Figure B.4: Cumulative distribution plot of adapted microwave-assisted derivatisation conditions. Amount of organic acids (%) and the CV (%) obtained for the organic acids after derivatisation and GC-MS analysis.