tert-Butyldimethylsilyl as an alternative for Trimethylsilyl derivatisation during organic acid analysis: a comparison

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ABSTRACT

One of the prevailing fundamental analyses for diagnosing patients with presumable inherited metabolic diseases is organic acid analysis which has the ability to diagnose a number of various diseases within a single run. TMS (Trimethylsilyl) derivatisation remains the most widespread method of derivatisation during organic acid analysis. Ion fragment intensities of TMS derivatives remain small, however, causing quantification to be restricted by MS (mass spectra). An alternative derivatisation method is tBDMS (tert-butyldimethylsilyl) derivatisation which works with the same principle as TMS derivatives. The derivatising reagent tBDMS has advantages over agents that generate TMS derivatives in that tBDMS has higher reactivity and generates moisture stable derivatives with larger [M-57] ions that confer higher detection sensitivity by MS. In this study, TMS derivatives and tBDMS derivatives were compared to one-another using a number of representative organic acid standards. These representative organic acid standards were derivatised with both TMS and tBDMS respectively and run on a GC-MS (Gas Chromatography-Mass Spectrometry) to investigate whether there is a difference in their mass spectra, on-column stability, and precision. Furthermore, after dervatisation, second phase separation was performed with tBDMS dervatisation to investigate whether tBDMS derivatisation will diminish other non-organic acid compounds more efficiently. Literature suggests that tBDMS derivatives tend to be superior to TMS derivatives. This study concurs with the above-mentioned declaration as tBDMS proved to be the predominant derivative in organic acids analysis. Furthermore, tBDMS derivatisation with second-phase separation did to some extent reduce the background noise, but further investigation is recommended for future studies.

KEY TERMS: Organic Acids, Derivatisation Comparison and GC-MS analysis.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
AMDIS	Automatic Mass Spectral Deconvolution and Identification System
BSTFA	Bistrimethylsilyltrifluoroacetamide
CV	Coefficient of variation
EIC	Extracted ion Chromatogram
ERNDIM	European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism
GC	Gas Chromatography
IS	Internal Standard
m/z	mass to charge ratio
MS	Mass Spectrometry and can also represent Mass Spectrum
MSTFA	N-methyltrimethylsilyltrifluoroacetamide
MTBSTFA	N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide
NIST	National Institute of Standards and Technology
ppm	Part Per Million
QC	Quality Control
tBMDS	tert-Butyldimethylsilyl
TIC	Total Ion Chromatogram
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl

CHAPTER 1: PREFACE

1.1 BACKGROUND AND PROBLEM STATEMENT

The need to analyse increasingly smaller amounts of compounds has grown exponentially. Therefore, it has become paramount to extend the range of detectability of the compounds in question. Improvement in detectability and method sensitivity can be accomplished through derivatisation that creates a surrogate for the original compound while increasing the mass, or when functional groups or atoms are introduced, that interact strongly with the detector. For example, with gas chromatography (GC) the formation of Trimethylsilyl (TMS) derivatives produces identifiable mass ions and identifiable fragmentation patterns (Ohie *et al.*, 2000). For GC analysis, derivatisation is often required to make compounds more suitable (volatile) for analysis. Moreover, well-selected dervatisation procedures also improve chemical separations, based on the target compounds' chemical composition (Halket & Zaikin, 2003).

Derivatisation is a technique that generally requires a reaction between the analyte(s) in question and a specific derivatisation reagent. There are numerous derivatisation reagents available for use, depending on the functionality and character of the analyte(s). The addition of a TMS functional group to the compound, also referred to as Trimethylsilylation, is generally used in GC analyses. Information regarding the chemical structure of compounds are often revealed through the mass spectra of TMS derivatives. A drawback is that the intensities of particular ion fragments remain small, causing the quantification sensitivity to be restricted by mass spectrometry (MS). Another widely used derivatisation reagent is tBDMS (tert-Butyldimethylsilyl) due to the fact that The derivatising reagent tBDMS has advantages over agents that generate TMS derivatives in that tBDMS has higher reactivity and generates moisture stable derivatives with more intense [M57] ions that confer higher detection sensitivity by MS. A comparison between TMS and tBDMS derivatives would therefore be of value.

Having an appropriate derivatising procedure and reagent should produce the required chemical modification of the compound(s), and be reproducible, non-hazardous and efficient. Comparisons of cost, recovery, variation and availability between tBDMS and TMS derivatisation will be made, along with chemical modifications and hazards.

1.2 STRUCTURE OF DISSERTATION

Abstract: Brief summary of the main focus points and conclusions of the study.

Chapter 1: Short background of the theoretical aspects of the study and also the structure of the dissertation.

Chapter 2: Literature review. This will include an in-depth discussion of the existing literature concerning the theme and key topic points of the study.

Chapter 3: Aim, objectives, scope and substantiation. This chapter will outline the problem statement, aim, objectives, scope and substantiation of the study.

Chapter 4: Materials and Methods. All materials used will be listed fully. The method section will contain the experimental design of the study, which will include the methodology, collection and analytical procedures used.

Chapter 5: Results and Discussion. Will include all important results obtained in the study and an in-depth discussion of all the results obtained and if all objectives were attainable.

Chapter 6: Conclusion. Will describe the most significant conclusions made during the study and future recommendations.

Bibliography

Addendums

CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION

Organic acid analysis is one of the leading analyses for diagnosing patients with presumable inherited metabolic diseases, including organic acidemias. Organic acidemias will more often than not be analysed through urine samples, because most organic acids are well discarded from blood by the kidney (Chalmers, 1982). The diagnosis and therapy of inborn errors of metabolism depend heavily on the detection and identification of organic acids in urine (Lo *et al.*, 2010). The term organic acids can be described as low molecular weight, water-soluble carboxylic acids which are either intermediates or products belonging to the metabolism of amino acids, carbohydrates, lipids, or biogenic amines (Kumps *et al.*, 2002).

The most common protocol used for organic acid analysis, despite newer methods, entails the extraction of acidified urine with organic solvents, derivatisation by means of silylation and finally the gas chromatographic separation and mass spectrometric detection of organic acids (Woontner & Goodman, 2006) as early as reported in Tanaka *et al.* (1980a). The main purpose of the extraction of organic acids is to simplify the matrix and reduce the matrix effects so that the sample is pre-concentrated to improve the sensitivity and selectivity of GC-analysis (Hintikka, 2018). When compounds are injected by GC-MS and move through the column, the more volatile compounds will elute first due to the temperature still being low. Compounds with higher boiling points (least volatile compounds) will elute much later as the temperature increases (Greaves & Roboz, 2013). Compounds enter the MS source by way of a heated transfer region. Compounds are then ionized through fragmentation and detected according to their mass-to-charge ratio (m/z) and identified (Greaves & Roboz, 2013). The need to analyse increasingly smaller amounts of compounds has grown exponentially. Therefore, it has become paramount to extend the range of detectability of the compounds in question. Through the process of derivatisation, the detectability of compounds increases, which enhances the sensitivity of the method.

2.2 DERIVATISATION

Derivatisation refers to the chemical process where compounds are modified to form new products that have better chromatographic properties (Schummer *et al.*, 2009). Before samples can be analysed by GC, derivatisation is needed to make a sample more suitable for analysis. Molecules that have the following functional groups have the ability to form hydrogen bonds between compounds: -OH, -SH, -COOH and -NH. Without the ability of derivatisation, molecules

with the above-mentioned functional groups will have weak volatility, insufficient stability, and may generate interactions of the compounds with the solid column leading to low detectability (Schummer *et al.*, 2009).

Ideally, a derivatising reagent should include an adequate procedure and an adequate derivatising reagent (Sellers, 2010). There are general criteria that will indicate what the ideal derivatisation reagent must look like. Firstly, a majority of complete derivatives must be produced from the reagent. Essentially in the process of derivatisation, there should be no structural change or rearrangements of the compounds. After derivatisation, the derivative's structure should be close to the compound's original non-derivatised structure (Moldoveanu & David, 2018). Additionally, there must be no contribution to sample loss. Ultimately, a derivative must be produced that will not permanently interact with the GC column and which is stable with regard to time (Orata, 2012).

There are three basic types of derivatisation reactions pertaining to GC, which include silylation, acylation and alkylation (Sellers, 2010). It is beyond the scope of this study to specify the acylation and alkylation reactions, thus silylation will be the only reaction to be explicated.

2.3 SILYLATION AND DERIVATIVES

Silylation currently remains one of the most versatile procedures for derivatisation (Parkinson, 2012). Silylation can be described as the chemical reaction that replaces active hydrogen atoms on -OH, -SH, -NH and -COOH groups, mostly with Trimethylsilyl (TMS) group [-SI(CH_3)$_3$] (Halket & Zaikin, 2003). A simplified general silylation reaction is seen in Figure 2-1. Since silylated derivatives tend to be more stable and more volatile, peaks are produced that are narrow and symmetrical. Additionally, silyl derivatives have improved mass spectral properties as silyl groups enable distinctive fragmentation pathways in order to reveal the structure of a compound and abundant characteristic ions in the spectra of these derivatives (Halket & Zaikin, 2003). Silylation is mainly carried out to reduce the polarity of the analyte in chromatography, and also to improve the GC behaviour (Moldoveanu & David, 2018). It is important, in silylation derivatisation, that both the sample and solvents are dry (Sobolevsky *et al.*, 2003), because any form of water entering the samples will lead to hydrolysis of the silylating reagent, and therefore any targeted analytes will be prevented from undergoing derivatisation (Sellers, 2010).

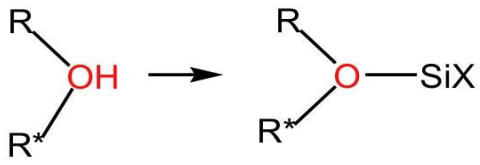


Figure 2-1: Simplified general silylation reaction (adapted from Kataoka, 2005). R and R*; alkyl, aryl or hydrogen. X; TMS or tBDMS.

When an analyte is not soluble in the derivatising reagent, solvents are commonly used. Solvents solubilise both compounds and the derivatisation reagent (Nicholson, 1978a). Some of the solvents that can be used include pyridine, dimethylformamide, acetonitrile, dimethyl sulphoxide, chloroform, toluene and carbon disulphide. All solvents have some degree of catalytic activity (Nicholson, 1978a). Pyridine is the most widely used reaction medium, as its catalytic ability increases the reactivity of a reagent more so than compared to the other solvents mentioned (Nicholson, 1978a). Pyridine will drive the reaction forward as it is an acid scavenger (Orata, 2012). These solvents lack functional groups with active hydrogen atoms (Halket & Zaikin, 2003) so they do not interact with the silylation reagent. The solvent volume must be as little as possible and the solvent must be very pure (Sobolevsky *et al.*, 2003). By using small amounts of pure solvent, unnecessary peak formation will be prevented (Moldoveanu & David, 2018).

The ease of reactivity of the functional group concerning silylation will be: Alcohol > Phenol > Carboxyl > Amine > Amide/hydroxyl (Orata, 2012), where the order pertaining to alcohols is Primary > Secondary > Tertiary. The same order applies to amines (Kataoka, 2005).

2.3.1 REAGENTS USED IN SILYL DERIVATISATION

There are numerous reagents available for silyl derivatisation, of which only a few of the most widely used reagents will be mentioned and discussed. Trimethylchlorosilane (TMCS), Bistri-methylsilyltrifluoroacetamide (BSTFA), N-methyltrimethylsilyltrifluoroacetamide (MSTFA) are the most common reagents used in silylation (Parkinson, 2012). Another reagent used for silylation is N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA), although it is not the most prevalent reagent for usage. The reason is likely due to the derivatisation reagent being chosen based largely on the availability of libraries, cost of reagent, suitability, and the efficiency of derivatisation that occur. These silyl reaction conditions have considerable differences and are dependent on the reactivity of the derivatised group and the degree of steric hindrance it is subjected to (Nicholson, 1978b).

2.3.1.1 Trimethylchlorosilane

When derivatisation is done by using TMCS, TMCS produces a by-product HCL, which is acidic and thus makes it infrequently used (Orata, 2012). Attributable to this, the reagent is not used regularly and is rarely used alone, as derivatisation may not be completed (Halket & Zaikin, 2003). On the other hand, when used in conjunction with another silyl reagent it acts as a catalyst and increases the reactivity of the other silyl reagent. An example of how the general derivatisation reaction occurs can be seen in Figure 2-2.

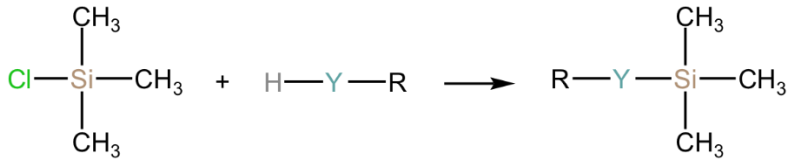


Figure 2-2:TMCS silylation reaction. Y: O, NH, S, COO. R: Alkyl group and/or Aromatic rings. This figure was adapted from Orata (2012).

2.3.1.2 Bistrimethylsilyltrifluoroacetamide

Bistrimethylsilyltrifluoroacetamide (BSTFA) is one of the reagents that is considered the most popular silylation type used for derivatisation. Bistrimethylsilyltrifluoroacetamide reacts faster and more completely, whereas additionally, its by-products don't interfere with the resulting spectrum as they are extremely volatile (Parkinson, 2012). To guarantee the completion of the reaction, it can be heated for 5 to 10 minutes at 60ºC (Orata, 2012). Figure 2-3 shows the general reaction of BSTFA silylation.

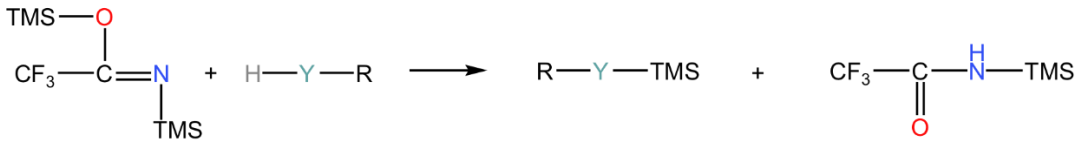


Figure 2-3: **BSTFA silylation reaction.** TMS: $Si(CH3)_3$. Y: O, NH, S, COO. R: Alkyl group and/or Aromatic rings. This figure was adapted from Orata (2012).

TMCS is often added to the reaction, along with BSTFA, to increase the stability of the reaction by catalysing functional hindered groups and secondary alcohols and amines. (Orata, 2012) (Parkinson, 2012).

2.3.1.3 N-methyltrimethylsilyltrifluoroacetamide

Of all the TMS acetamides, MSTFA is the most volatile (Moldoveanu & David, 2018), due to its by-product *N*-methyltrifluoroacetamide which is more volatile than BSTFA's by-products (Moldoveanu & David, 2018). There are many advantages with the use of this technique over conventional approaches such as a minimum solvent that is needed for derivatisation. Furthermore, the reaction is fairly mild and doesn't require increased heating (Nicholson, 1978). The silylating reaction for MSTFA can be seen in Figure 2-4.

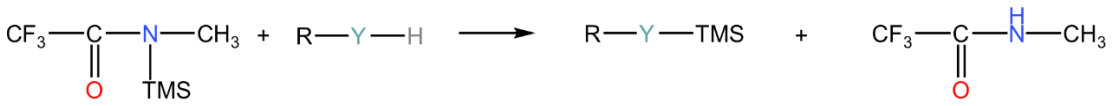


Figure 2-4: MSTFA silylating reaction. TMS= $Si(CH3)_3$. Y: O, NH, S, COO. R: Alkyl group and/or Aromatic rings. This figure was adapted from Orata (2012).

Nonetheless, the use of this reagent will only be appreciated when working compounds that elute early as these TMS derivatives are more volatile (Nicholson, 1978).

2.3.1.4 N-methyl-N-t-butyldimethylsilyltrifluoroacetamide

Using MTBSTFA as a derivatisation reagent works by replacing the active hydrogen with a tBDMS ([-$Si(CH_3)_2C_4H_9$]) group. tBDMS derivatives are up to 10 000 times more stable than TMS derivatives and more hydrolysis resistant (Orata, 2012). Derivatives containing tBDMS moieties or MTBSTFA generate easily interpreted mass spectra. Figure 2-5 shows the general silylation with MTBSTFA.

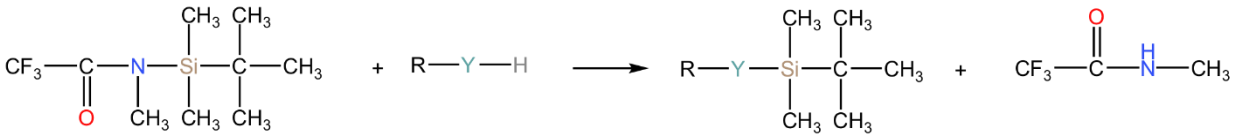


Figure 2-5: MTBSTFA silylation reaction. Y: O, NH, S, COO. R: Alkyl group and/or Aromatic rings. This figure was adapted from Orata (2012).

The literature contains comprehensive comparisons of overall GC-MS methods, but in hindsight, there is no comprehensive comparison of the different derivatisation methods, which makes it difficult to deduce an optimal derivatisation method.

2.3.2 Chromatographic properties of silyl derivatives

When looking at the chromatographic properties of silyl derivatives, a dimethyl-siloxane polymer with 5% phenyl group substitutions is the most popular stationary phase and is acceptable for the majority of analytes. Depending on the nature of the analytes, different specialized phases may be utilized; for example, alumina plot columns are used for gases and organic molecules with low molecular weights (less than 100 Da). Numerous chemicals can be promptly separated using the widely utilised and highly effective fused silica capillary columns (Greaves & Roboz, 2013). Furthermore, low polarity phases are frequently used, and hydroxyl-containing phases like the different Carbowaxes must be avoided since too much reagent will react with those phases (Nicholson, 1978b). Columns developed and applied with covalently bonded and cross-linked phases give the ability of the upper limit of the working temperature to be raised, which facilitates compounds that needs to be analysed having a higher molecular weight, silylated polyfunctional and compounds with heavy silyl groups (Halket & Zaikin, 2003).

2.3.3 Mass spectral properties of silyl derivatives

Silyl derivatives have many resourceful aspects, as it is widely used in mass spectral analysis. For example, it can be used for identification or structure elucidation together with screening, profiling and quantitative determination of various organics (Halket & Zaikin, 2003). Identifying the organics can ultimately be done by using reference databases and mass spectral libraries. There are several software available that compare the recorded and reference spectra, with automatic identification, if the spectrum of a target compound is present in the library. One of the widely used available software is the Automatic Mass Spectral Deconvolution and Identification System (Stein, 1999), more commonly referred to as AMDIS (Qiu & Reed, 2014).

2.4 TMS AND tBDMS DERIVATIVES AND THEIR PROPERTIES.

2.4.1 TMS derivatives

The addition of a TMS functional group to the compound, also referred to as Trimethylsilylation, is the most widely used derivatisation reagent in GC-analyses (Moros *et al.,* 2017).

The mass spectral features of TMS derivatives are characteristic, namely that a characteristic fragment ion is formed during EI (Electron ionisation) that produces a molecular ion [M-15] for TMS derivatives, resulted by the neutral loss of a methyl group (Gross, 2004), albeit at low abundances (Harvey & Vouros, 2020). Incomplete silylation can result in the formation of multiple peaks, evidently forming one, two, three or more TMS groups of a specific compound (Fritsche-Guenther *et al.*, 2021).

Withal, TMS derivatives show good separation on the chromatographic column (Kałużna-Czaplińska, 2011). However, TMS derivatives are unstable under humid conditions and are liable to temperature changes and fragmentation (Kałużna-Czaplińska, 2011). Additionally, it was reported by Ohie *et al.* (2000) that particular [M-15] ions have ion intensities that remain small, causing the quantification sensitivity to be restricted by mass spectrometry.

2.4.2 tBDMS derivatives

Derivatisation with tBDMS is also a widely used derivatisation reagent, but less popular than TMS (Hintikka, 2018; Nguyen *et al.*, 2013), even though tBDMS derivatives are stable against moisture (that includes being stable in water) have higher reactivity and a more intense molecular ion [M-57], resulting from the neutral loss of a butyl group, compared to TMS derivatives (Halket & Zaikin, 2003; Moldoveanu & David, 2018; Schummer *et al.*, 2009). Consequently, tBDMS derivatives have improved sensitivity for quantification than TMS derivatives when a high molecular mass ion is desired for quantification (Hintikka *et al.*, 2008; Ohie *et al.*, 2000).

The expected results of using MTBSTFA rather than TMS is that there will be increased hydrolytic stability and improved sensitivity (Poole & Zlatkis, 1979). When using silyl derivatives other than TMS, it is important to keep in mind that the molecular weight will increase. Thus, polyfunctional compounds will form that will result in retention times that strongly increase (Halket & Zaikin, 2003). On the other hand, it can be advantageous when compounds with low molecular weights need to be analysed (Halket & Zaikin, 2003), as some organic acids present with significant difficulties in identification because they might not have been completely extracted prior to derivatisation or they occur in small amounts (low concentrations). Identification of 4-Hydroxybutyric acids' TMS derivative may be missed in patients who excrete tiny amounts of this organic acid, as it elutes close to the urea peak. Succinylacetone is a supplementary example of a compound that may be misidentified when small amounts are present in urine or may disappear in alkaline solutions (Kałużna-Czaplińska, 2011). Succinylacetone is a vital marker of Tyrosinemia type I, which makes the identification of this compound substantial.

2.4.3 Mass spectral properties of TMS derivatives

Fragmentation properties of organic acids as their TMS derivatives differ from sub-classes of organic acids. The mass spectral properties for the different sub-classes of organic acids as their TMS derivatives will be discussed below, apart from their most occurring [M-15] ion.

2.4.3.1 Aliphatic Carboxylic acids:

Long-chain fatty acids, e.g., stearic acid (mono-carboxylic acid) are expected to show m/z 73, 75, 117, 129, 132 and 145 m/z as characteristic fragment ions in their mass spectrum. Dicarboxylic acid and Tricarboxylic acids have characteristic fragment ions, with high intensities, that are commonly observed in their MS. These fragment ions are 73 m/z and 147 m/z. (Okahashi *et al.*, 2019).

2.4.3.2 Benzoic acids (including sub-classes of Benzoic acids like Hippuric acid)

Benzoic acids produce characteristically high-intensity fragment ions namely 77, 105 and m/z 135 (Harvey & Vouros, 2020).

2.4.3.3 Bifunctional Compounds (Hydroxy-carboxylic acids)

The other major fragment ions, apparat from the abundant [M-15] that are commonly observed in their mass spectrum, are m/z 73, 131 and 147m/z. These fragment ions have high intensities making these fragment ions characteristic of Hydroxy-carboxylic acids (Harvey & Vouros, 2020).

2.4.3.4 Keto acids

Some of the characteristic ions observed for Keto acids are 73 m/z and 147 m/z (Nguyen *et al.*, 2013).

2.4.4 Mass spectral properties of tBDMS derivatives

Fragmentation patterns for tBDMS seem to not have been studied extensively enough to obtain conclusive mass spectral properties about the general fragmentation patterns of tBDMS derivatives in different sub-classes of organic acids. Below the most common ions that are suspected to be found in tBDMS derivatives of organic acids, other than the most occurring [M-57] ion, will be discussed according to sub-classes of organic acids.

2.4.4.1 Carboxylic acids:

Long-chain fatty acids, e.g., stearic acid (mono-carboxylic acid), are expected to display the following fragment ions: 75 m/z (Harvey & Vouros, 2020, Kim *et al.*, 1989), 73, 117 129 and 131 m/z (Kim *et al.*, 1989). The major fragmentation ions of Dicarboxylic acid and Tricarboxylic acids are expected to be 73, 75, 115, 117, 129, 133, 147 and 189 m/z (Mawhinney *et al.*, 1986).

2.4.4.2 Benzoic acids (Including sub-classes of Benzoic acids like Hippuric acid)

The main fragment ions to be expected, with high intensities included, are 77 m/z and 105 m/z. Other fragments also to be expected, but with lower intensities, include 51, 73, 75, 135 and 192 m/z (Kim *et al.*, 1989).

2.4.4.3 Bifunctional Compounds (hydroxy-carboxylic acids)

Major fragment ions, with high intensities, that are expected to be found in the MS of hydroxyl-carboxylic acids are 73, 133, 147 and 189 m/z, whereas the minor fragment ions expected to be found is 65 m/z, 75 m/z, 91 m/z and 137 m/z, with very low intensities (Kim *et al.*, 1989).

2.4.4.4 Keto acids

Major fragment ions that are expected to be seen in the mass spectra of Keto acids are 73 m/z and 147 m/z, with varying lower-intensity fragment ions that are expected in the MS. (Nguyen *et al.*, 2013).

2.4.4.5 Conclusion

The preceding sections in this chapter have provided a discussion pertaining to the preparation and identification of organic acids. An alternative derivatisation method (MTBSTFA), along with BSTFA, was also discussed regarding their stability, mass spectral properties and quantification sensitivity. There is limited recent literature concerning a well-defined comparison between TMS derivatives and tBDMS derivatives with regards to the above-mentioned discussion points in urinary organic acid analysis. Previous literature that conducted a comparison between TMS and tBDMS derivatives include (De Jong *et al.*, 1980; Harvey & Vouros, 2020; Kałużna-Czaplińska, 2011; Mawhinney *et al.*, 1986; Ohie *et al.*, 2000; Okahashi *et al.*, 2019; Schummer *et al.*, 2009). Overall, these publications concluded that tBDMS derivatisation produces an abundant M-57

fragment ion that is more suited for quantification when compared to the M-15 TMS derivatisation ion. Recovery and variation from tBDMS derivatisation were superior to TMS as is to be expected given the abundance of the M-57 ion. None of the studies assessed the stability of the TMS and tBDMS derivatives while awaiting or during analysis.

Considering the above-mentioned limitations, it would be valuable to assess the mass spectrometric properties of organic acids by examining the completeness of the derivatisation reaction in terms of derivatisable groups. Furthermore, a comparison between stability and precision between TMS and tBDMS would also be of value.

CHAPTER 3: AIM, OBJECTIVES, SCOPE AND SUBSTANTIATION

3.1 Introduction

One of the prevailing fundamental analyses for diagnosing patients with presumable inherited metabolic diseases is organic acid analysis, which has the ability to diagnose a number of various diseases within a single run. Derivatisation through TMS remains the most widespread method of derivatisation in organic acid analysis. As the demand for quantitative organic acid analysis increases, TMS may not be the most suitable derivatisation technique. Additionally, TMS derivatives show good separation on the chromatographic column (Kałużna-Czaplińska, 2011), yet TMS derivatives are unstable under humid conditions and are liable to temperature and fragmentation (Kałużna-Czaplińska, 2011). However, tBDMS has been described as a suitable alternative to TMS due to the derivatising reagent tBDMS having advantages over agents that generate TMS derivatives in that tBDMS has higher reactivity and generates moisture-stable derivatives with larger [M-57] ions that confer higher detection sensitivity by MS. tBDMS derivatisation ultimately has the ability to extend the range of detectability, which is paramount in GC-MS organic acid analysis.

3.2 AIM

This study aims to investigate tBDMS as a suitable alternative to TMS dervatisation during organic acid analysis. tBDMS dervatisation may be more suited for quantification due to the presence of a high molecular mass fragment of high intensity [M-57] and increased stability due to resistance to hydrolysis.

3.3 OBJECTIVES

- Familiarization with organic acid mass spectra and confirmation of fragmentation pattern as described in the literature. 13 Representative organic acid standards will be made up individually in methanol. All organic acid standards will be injected individually for both TMS and tBDMS derivatives, to see differences in their individual spectra. For some of the compounds, only one peak is possible and for all these compounds only one peak is expected to be seen unless: dervatisation was incomplete, structural isomer formed, Tautomerism occurred, and/or partial hydrolysis after dervatisation occurred. Additionally, the mass spectrum of each organic acid standard for both TMS and tBDMS derivatives

will be compared. When organic acids are derivatised, the possibility remains that a compound can produce one to three or more TMS/tBDMS groups, depending on the compound in question, and thus possesses the possibility of more than one unique high fragment [M-15] and/or [M-57] ion that can be yielded. Considering this, all theoretically possible mass fragment [M-15] and/or [M-57] ions will be explored to obtain the primary [M-15] and/or [M-57] fragment ions along with the examination of all characteristic fragmentations patterns. This will be done by examining all possible TMS and tBDMS groups that can derivatise on a compound in question. The identification of these groups can be challenging, and therefore characteristic fragment ions that are expected to show on a specific organic acid profile will be used for confirmation purposes.

- The mass spectrum will be compared by comparing the abundances of the primary [M-15] and/or [M-57] ions found. This will be achieved by comparing the abundance of the M-15 (TMS derivative) and M-57 (tBDMS derivative) ions for each organic acid. Evaluating the abundance of the fragment ions for both the derivatisation methods enables the assessment of quantification sensitivity.

- Compare the stability of derivatisation for both TMS and tBDMS derivatives. Partial derivatisation may be problematic if the percentage contribution of the partially derivatised peak is unpredictable, as well as where only one peak is desired for quantification. The stability, or more specifically the stability during the waiting period in the autosampler and during injection/volatilisation for both derivatisation methods will be evaluated, by injecting TMS derivatised organic acids and tBDMS derivatised organic acids spiked in synthetic urine over 24 hours. Having information regarding the stability of the two derivatising methods will be valuable to assess the number of samples that can be included in a batch.

- Compare the precision of the two derivatisation methods by using ERNDIM (European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism) and QC (Quality control) samples.

- Determine whether tBDMS derivatisation will reduce other non-organic acid compounds with an additional selectivity added. The additional selectivity is a second liquid-liquid extraction step carried out after derivatisation. This will serve as a second sample clean-up by adding water and hexane and extracting only the top layer (hexane layer – containing the silylated organic acids) for analysis. TMS derivatives are extremely sensitive to any water contamination including residual water vapour. On the contrary,

tBDMS derivatives are stable against moisture which includes being stable in water. With that being said, tBDMS derivatisation can improve the result outcome and thus only tBDMS derivatisation will be done for this determination.

3.4 SCOPE AND SUBSTANTIATION

Looking at the objectives of this study, using all organic acid standards was out of the scope and thus only representative organic acids were chosen for proof of concept. These representative organic acids were chosen in light of their functional groups. They were divided into two main types of organic acids; Carboxylic acids (Monocarboxylic acid, Dicarboxylic acids, Tricarboxylic acids), Benzoic acids, Bifunctional Compounds and Keto acids. These representative organic acids were chosen to see how the two dervatisation reagents will derivatise respectively on these 13 different structural compounds. The efficiency between BSTFA and MTBSTFA may differ in light of quantifiable peaks produced as a result of partial or no derivatisation that occurred. These 13 organic acids include a relatively broad spectrum of compounds to be analysed, as these compounds don't all have equal derivatisation sites, molecular masses or functional groups, which makes these chosen standards sufficient to study the differences between them. The 13 representative compounds are listed in Table 3-1.

In light of the aim of this study, the focus was removed from the extraction step as well as the instrumental optimization, as dervatisation was the main objective that was being researched. The reason for only focusing on the dervatisation of organic acids is so that the conditions otherwise are kept constant, and thus a more accurate comparison in the two derivatisation methods can be examined.

In addition, it was decided to omit oximation from the process, as organic acid analysis is usually done without oximation and only done when oxo-acids are expected, e.g., Succinylacetone. A patient with Tyrosinemia can easily be missed seeing that Succinylacetone is prone to degradation (Kałużna-Czaplińska, 2011). Because of the increased stability of tBDMS, tBDMS derivatisation may be better for flagging results that will require reanalysis after oximation.

Hippuric acid is classified here as a Benzoic acid, but it is also a Glycine conjugate, albeit low concentrations of glycine conjugates that are frequently measured during OA analysis. Glycine conjugates are problematic due to poor extraction efficiency, multiple peak formation, and it has also been suggested that glycine conjugates are measured using a separate method (Rinaldo, 2008).

Furthermore, considering the scope of the objectives, all compounds were analysed through scan mode only, as there was a desire to compare the findings to an in-house method. There was also some uncertainty concerning hydrolysis and/or isomerism prior to analysis.

Moreover, choosing the best derivatisation method to be used in this study proved to be very difficult, as it became noticeable during the search for the best method that numerous other laboratories have some differences in their extraction and derivatisation procedure, even if they follow the same extraction and derivatisation procedure to some extent. Looking at some of the laboratories doing organic acid analysis, these differences include the internal standard (IS) used as well as the incubation time after derivatisation. The internal standards range from Dimethylmalonic acid, Tropic acid, Pentadecanoic acid (Metz, 2011), a mixture of 11 stable-isotope-labelled compounds in methanol (Blau *et al.*, 2008), a mixture of Margarate, Tetracosane and Tropate (Ohie *et al.*, 2000), Pentadecanoic acid (Christou *et al.*, 2014), and also Tropate and Heptadecanoic acid (Sheng & Wang, 2017). The incubation time after derivatisation ranged from 50°C - 85°C for 15 - 30 minutes. The similarities that are found in the extraction and derivatisation procedures are the acidification and extraction reagents, the dehydration and drying of the samples, and the derivatising reagent (Blau *et al.*, 2008, Christou *et al.*, 2014, Metz, 2011, Sheng & Wang, 2017) with Ohie *et al.* (2000) being the exception that used MTBSTFA rather than BSTFA + 1%TMCS. The only difference here was the amount of reagents used.

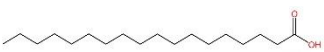
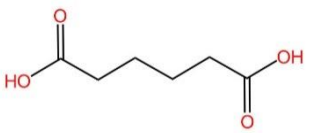
Pyridine was chosen, as there are currently no gold standard and different labs use slight modifications of the original method proposed by Tanaka *et al.* (1980a), and Tanaka *et al.* (1980b). Additionally, tBDMS derivatisation was desired to be compared to the in-house method. The scarcity of literature available suggests that tBDMS is preferable for quantification, as discussed thoroughly in Chapter 2, and the tBDMS derivatisation protocol that is most in line with the in-house method was selected from literature (Kałużna-Czaplińska, 2011; Nguyen *et al.*, 2013).

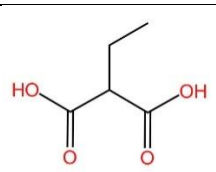
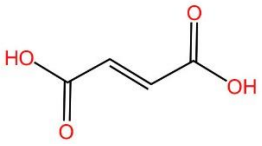
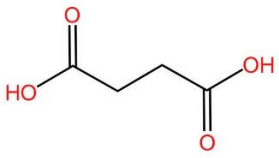
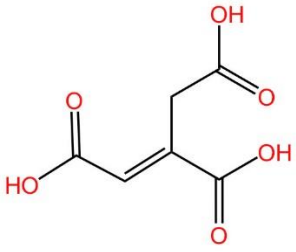
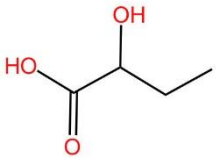
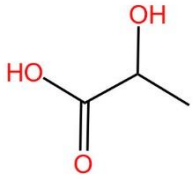
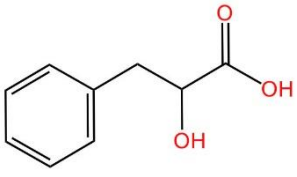
This study is a proof of concept and is not intended to be a full comparison. Ideally, it is desirable to assess accuracy as well, but was not done for the proof of concept, seeing that the assigned values of the material that was used may not be accurate. This is because values are often assigned based on concentrations obtained by using a typical method or a group average which can be expected to be mostly TMS methods. Furthermore, seeing that most methods quantify organic acids using an internal standard only, quantification is relative at best and not necessarily linear over the reported range. Lastly, imprecision was regarded as the most important quality

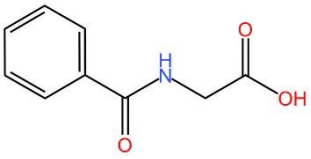
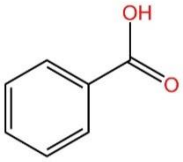
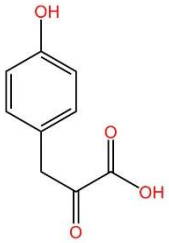
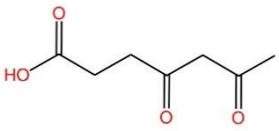
parameter, seeing that biases can be corrected with several methods including the use of response factors and calibrator concentration reassignment.

Additionally, non-organic acid compounds may develop during the derivatisation process, which can lead to the background noise that increases during GC-MS-analysis. Background noise can lead to signal degradation along with reduced signal-to-noise ratios, and influence the skewness of the peak shapes (Chua *et al.*, 2017). When other non-organic acid compounds can be reduced before GC-MS-analysis, the aforementioned problems can be minimised, ultimately making the interpretation of mass spectrums more facile. This can be achieved by introducing a second-phase separation, more formally known as a biphasic separation, after derivatisation (Araujo et al., 2008). The original biphasic separation procedure was done on fatty acid methyl esters, where water and hexane were added after the extraction and derivatisation procedures were completed (Araujo et al., 2008). The proposed experimental design will to some extent remain the same for organic acids, where the procedure entails the addition of water and an appropriate apolar organic solvent (hexane). This will form a two(bi)-phase system, which will purify the extracted organic acids (Najafabadi et al., 2014). This allows for all polar and neutral organic acids to be partitioned in the organic phase, while other non-organic acid compounds, will be partitioned in the water phase (Najafabadi et al., 2014) (Halim et al., 2012).

Table 3-1: All representative organic acid structures with their derivatised masses.

	Compound name	**Structure**	**TMS Derivatised Mass (g/mol)**	**tBDMS Derivatised Mass (g/mol)**
Monocarboxylic acids	1. Stearic acid		356.472	398.562
Dicarboxylic acids	2. Adipic acid		290.458	374.638

	3. Ethylmalonic acid		276.442	360.622
	4. Fumaric acid		260.411	344.591
	5. Succinic acid		262.427	346.607
Tricarboxylic acids	6. Aconitic acid		390.616	516.886
Bifunctional compounds	7. 2-Hydroxbutyric acid		248.447	332.627 g/mol
	8. Lactic acid		234.432	318.612
	9. 3-Phenyllactic acid		310.463	394.643

Benzoic acid compounds	10. Hippuric acid		323.338	347.638
	11. Benzoic acid		194.236	236.326
Keto acids	12. 4-Hydroxyphenyl pyruvic acid		324,45	522,9
	13. Succinylacetone		230.258	272.348

All figures that are displayed in Table 3-1, were adapted from the organic acid figures from The Human Metabolome Database.

CHAPTER 4: MATERIALS AND METHODS

4.1 Introduction

Derivatisation is an essential step during sample preparation of organic acid analysis. Derivatisation is often required before GC-MS-analysis to make compounds suitable for analysis, by reducing the polarity and increasing the volatility of compounds. As a result, derivatisation ultimately ameliorates the spectra obtained after analysis. It is thus imperative to find a suitable derivatisation reagent that will ensure consistent results. Consequently, it was necessary to compare two derivatisation procedures to certify consistent results. With this in mind, a detailed description of this chapter will be discussed in accordance with: chemicals and reagents, preparation of solutions, GC-MS-parameters, sample preparation and derivatisation, and finally data processing.

All methods used in this study were procedures previously done by an in-house method as well as other laboratories (which served as a reference), with minor modifications. (Christou *et al.*, 2014, Kałużna-Czaplińska, 2011, Ohie *et al.*, 2000). These minor modifications had no impact on the integrity of the data that were obtained for both TMS and tBDMS derivatisation methods.

4.2 Chemicals and reagents.

The following organic acids and reagents were purchased from Sigma-Aldrich (CAS no): 2-Hydroxbutyric acid (3347-90-8), 3-Phenylbutyric acid as Internal Standard (4593-90-2), 3-Phenyllactic acid (828-01-3), 4-Hydroxyphenylpyruvic acid (156-39-8), 5N HCl (7647-01-0), Aconitic acid (4023-65-8), Adipic acid (124-04-9), Benzoic acid (65-85-0), Diethyl ether (60-29-7), Ethyl acetate (41-78-6), Ethylmalonic acid (601-75-2), Fumaric acid (110-17-8), Hippuric acid (495-69-2), Hexane (110-54-3), Lactic acid (50-21-5), Methanol (67-56-1), Pyridine (110-86-1), Stearic acid (57-11-4), Sodium sulphate (7757-82-6), Succinic acid (110-15-6), Succinylacetone (51568-18-4), and Synthetic Urine (Lot # 72124). Dervatisation reagents: TMCS (75-77-4), BSTFA (25561-30-2) and MTBSTFA (77377-52-7). The following reagents were bought from Honeywell: Distilled Water (7732-18-5). Isotopically labelled Orotic acid (Internal Standard) was bought from Cambridge Isotope Laboratories Inc. (NLM-1048-0-25).

4.3 Preparation of standards and samples

4.3.1 Individual organic acid stock and working solutions

An amount of 15 mg of each organic acid standard (fourteen organic acid standards) was weighed in a 15 ml polypropylene tube. 15 ml of Methanol was added to the polypropylene tube, which served as the individual stock solution having a concentration of 1000 ppm. 250 µl of individual stock solution was added to a 5 ml polypropylene tube. 4-Hydroxyphenylpyruvic, 3-Phenyllactic acid, 2-Hydroxybutyric acid, Aconitic acid, Adipic acid, Benzoic acid, Ethylmalonic acid, Fumaric acid, Hippuric acid, Lactic acid, Succinic acid, Succinylacetone and Stearic acid were all made up in methanol (4750 µl Methanol added to the 5 ml polypropylene tube), to a final concentration of 50 ppm and served as the individual working solution. The working solutions were then stored at -80°C.

4.3.2 Spiked synthetic urine sample

All individual stock solutions were taken out of the -80ºC freezer. Subsequently, 750 µl of each of the individual stock solutions were taken and combined into a 15 ml polypropylene tube, having a volume of 10,5 ml at this stage. 4,5 ml Methanol was added to the 15 ml polypropylene tube to have a final volume of 15 ml and a final concentration of 50 ppm which served as the organic acid standard solution. 1 ml of the organic acid standard solution was added to a kimax (15 ml) tube and dried under a gentle stream of nitrogen at 37°C. 1 ml Synthetic urine was then added to the dried sample and served as the spiked synthetic urine sample. The spiked synthetic urine sample was prepared each time on the day of the analysis.

4.3.3 Internal Standard (IS)

3-Phenylbutyric acid and Isotopically labelled Orotic acid were used to serve as the internal standard. A 26,25 mg/ml stock solution of 3-Phenylbutyric acid was prepared by dissolving 656,25 mg 3-Phenylbutyric acid in 1 ml ddH_2O and 1 drop 5N NaOH. The solution was made up to a final volume of 25 ml with ddH_2O using a volumetric flask. A 0,1725 mg/ml stock solution of Isotopically labelled Orotic acid was prepared by dissolving 8,625 mg Isotopically labelled Orotic acid:H_2O (1.3-15N_2) in 1 ml ddH_2O and 1 drop 5N NaOH. The solution was made up to a final volume of 50 ml with ddH_2O using a volumetric flask.

A working solution was prepared by adding 1 ml 3-Phenylbutyric acid stock solution and 2 ml Isotopically labelled Orotic acid stock solution to a 50 ml volumetric flask. A final volume of 50 ml was made up with ddH_2O.

4.3.4 ERNDIM and QC samples

ERNDIM samples (proficiency testing) were used and made up as instructed by ERNDIM with lot numbers; 2021.1471 and 2021.1472. QC samples were used and obtained from SKML with lot numbers; QC1: 2021.1471 and QC2: 2021.1472.

The ERNDIM scheme provides participant data for the following organic acids: 2-Hydroxyglutaric acid, 3-Methylglutaconic acid, 3-Methylglutaric acid, 3-Hydroxyisovaleric acid, 4-Hydroxybutyric acid, Adipic acid, Ethylmalonic acid, Fumaric acid, Glutaric acid, Glycolic acid, Methylmalonic acid, Pyroglutamic acid, Sebacic acid, Suberic acid and Tiglylglycine. To ensure sufficient volume, the ERNDIM samples were pooled and used as one sample for the rest of the preparation and extraction step. Furthermore, two QC samples were used (QC level 1 and QC level 2) with known organic acid compounds in the QC samples which were: 2-Hydroxyglutaric acid, 3-Hydroxy-3-Methylglutaric acid, 3-Hydroxyisovaleric acid, 3-Methoxy-4-Hydroxyphenyllactic acid, 3-Methylglutaric acid, 4-Hydroxbutyric acid, Adipic acid, Ethylmalonic acid, Fumaric acid, Glutaric acid, Glycolic acid, Malic acid, Methylmalonic acid, Tiglylglycine, Pyroglutamic acid and Sebacic acid.

4.4 Sample preparation and derivatisation

The procedure for sample preparation, analysis and data processing is thoroughly described in this section. The individual experimental designs are discussed in Section 4.6.1 to Section 4.6.4. Derivatisation with TMS and tBDMS was done in accordance with an in-house method and are in line with published methods (Christou *et al.*, 2014; Ohie *et al.*, 2000; Orata, 2012; Tanaka, *et al.*, 1980), with minor modifications.

4.4.1 Organic acid Extraction

Organic acid extraction was done according to the following procedure for all experiments that utilized spiked synthetic urine or ERNDIM/SKML material.

Six drops of 5N HCl (for acidification purposes) were added to the spike synthetic urine sample, which was in a 15 ml Kimax tube. 1 ml of the IS, as prepared in Section 4.3.3, was then added to the 15 ml Kimax tube. 6 ml Ethyl acetate was added to the 15 ml Kimax tube and mixed for 30

minutes on a rotary mixer, where after the sample was centrifuged at 3000 rpm for 3 minutes. This allowed for phase separation to occur. The organic phase (top layer) was transferred to a second 15 ml kimax tube using a glass Pasteur Pipette, after which 3 ml of diethyl ether was added to the remaining aqueous phase (bottom layer) and mixed for 10 minutes on the rotary mixer. The sample was centrifuged again at 3 000 rpm for 3 minutes. The second organic phase (top layer) was added to the previously collected organic phase in the appropriate tube (second 15 ml kimax tube). Approximately 3 g of anhydrous sodium sulphate (Na_2SO_4) was added to ensure that no water molecules were present. The hygroscopic nature of Na_2SO_4 ensures that any water molecules that are still trapped in the solvent phase, are removed. The sample was briefly vortexed and centrifuged again at 3 000 rpm for 3 minutes. The organic phase was then transferred to smaller kimax (10 ml) tubes and placed under a gentle stream of nitrogen at 37°C for approximately 45 minutes until the sample was dry.

No extraction was necessary when experiments were done using working solutions in methanol.

4.4.2 Derivatisation

After the samples were completely dry, they were silylated by adding derivatisation reagents to each sample, 45 µl BSTFA and 5 µl TMCS were added to the tubes labelled TMS. 50 µl MTBSTFA was added to the tubes labelled tBDMS. 50 µl Pyridine was then added to both TMS and tBDMS samples and vortexed briefly. The samples were incubated at 75°C for 45 min. After incubation, the samples were transferred to GC vials with inserts in them, ready for GC-MS-analysis. TMS and tBDMS derivatisation were according to the in-house procedure and in line with Gallagher *et al.*, (2018) and Kałużna-Czaplińska (2011), while tBDMS was done according to Ohie *et al.* (2000) with minor modifications.

4.5 GC-MS-parameters

An Agilent GC-system was used with an Agilent Technologies GC split liner (single taper, deactivated glass wool, low-pressure drop, 4 mm ID, 870 µL [part number 5183-4701]). Furthermore, a Restek GC Column was utilized (Rxi®-5silMS, 30 m x 0,25 mm x 0,25 µm capillary column [Serial number 1423746) as well as an Agilent Technologies gas clean filter system (part number CP17973).

Helium was used as a carrier gas at a constant column flow rate of 0,945 ml/s. A sample volume of one µl sample was used with a viscosity delay of 2 seconds, in split mode with a split ratio of 1:12, a split flow of 11,345 ml/min and the injector set to 280°C. An initial oven temperature of

50°C for 1 min which then increased to 60°C at 20°C/min, immediately followed by an increase to 120°C at 5°C/min, and finally to 280°C at 7°C/min, where it remained for 4 mins. The post-run temperature was set to 300°C for 1 min. There was a solvent delay of 7.00 mins, whereafter the GC-MS was set to scan from 50 – 600 m/z.

4.6 Data processing

Spectral data were annotated and quantified using AMDIS and Agilent Masshunter Qualitative Analysis. AMDIS enabled the identification of the organic acids, while Agilent Masshunter was used for comparative quantification of the extracted ion chromatogram (EIC) of the organic acids. The data were analysed according to the following settings in AMDIS: deconvolution settings, which included a component width of 32, adjacent peak subtraction at one, the resolution was high with sensitivity and shape requirements at low. Further settings included solvent tailing at 84 m/z and column bleed at 207 m/z.

When organic acids are derivatised, the possibility remains that a compound can acquire a variable number of TMS/tBDMS groups depending on functional or derivatisable groups of the compound in question. Consequently, certain organic acids can be detected as two or more separate peaks with different theoretical derivatised masses, and thus more than one expected TMS/tBDMS ([M-15]/[M-57]) peaks. Conventionally, a compound that had derivatised completely will yield the most abundant peak, over the other possible peaks that a compound can produce. Another possibility to consider is that the abundance of the other possible peaks can increase over time, while the abundance of the fully derivatised peaks can decrease. Furthermore, the possible derivatisable groups of TMS and tBDMS can be present in the individual standard solution and absent in the spike synthetic urine solution due to the different matrices used. In light of this, all expected TMS/tBDMS ions were explored for each representative organic acid in both the individual samples and the spiked synthetic urine samples (Table 5-1). The process for the above-mentioned went as follows: The structure of the compound was investigated to see how many places, theoretically, there are where derivatisation can take place. The derivatised mass for each compound was then calculated, as well as the [M-15] or [M-57] characteristic fragment ions, for TMS and tBDMS derivatives respectively. 2-Hydroxybutyric acid, for example, has a theoretical possibility of two TMS or two tBDMS groups forming on the compound, as seen in Table 5-1, after derivatisation. Thus, when there is only one TMS or one tBDMS group that is attached, the derivatised mass would be 176,25 g/mol and 218,34 g/mol for TMS and tBDMS respectively. The expected [M-15] and [M-57] for 2-Hydroxybutyric acid both for TMS and tBDMS will be 161. Not all compounds' [M-15] and [M-57] ions will be the same as in this example.

Furthermore, all possible [M-15] and [M-57] ions for each compound (due to variable attachment of TMS and tBDMS groups) were explored by searching for the expected [M-15] and [M-57] ions in the scan data generated for each standard. The relevant EICs were extracted in AMDIS, and peaks were examined. An identification was made by comparing the mass spectrum of a compound in question with the characteristic fragment ions found in an organic acid MS profile, as discussed in Section 2.4.3 and Section 2.4.4. Additionally, identification was confirmed by the NIST MS library if the applicable [M-15] and [M-57] were found in AMDIS. Table 5-1 contains all TMS and tBDMS derivative fragment ions that were explored to obtain the primary [M-15] and [M-57] ions of all TMS and tBDMS derivatives.

During the identification process of all possible derivatisable groups of TMS and tBDMS, an evaluation was made regarding EIC peaks that could be present continuously throughout the samples. If an unidentified peak was produced continuously throughout a sample, the term “Artifact” was used to describe any additional peaks not pertaining to the compound in question.

All organic acids were identified by means of mass spectral similarities using an in-house library as well as the NIST (National Institute of Standards and Technology) library. A new library was set up for the tBDMS derivatives in order to attain more accurate results. This was achieved by examining the mass spectrum for all individual organic acid standards in AMDIS. Their mass spectra were then matched with the NIST mass spectral library, and a library was created to identify the tBDMS derivatives more readily going forward and making data interpretation more comparable.

4.6.1 Individual organic acid standard preparations for individual chromatographic and MS assessment

Two batches were done for this assessment, one batch for TMS derivatives and the other for tBDMS derivatives, as 26 samples were too much for one batch. 50 µl of each of the individual organic acid standard working solutions were transferred to 14 x GC vials. The samples were dried under a gentle stream of nitrogen at 37°C for approximately 45 minutes. The samples were then derivatised as stated in Section 4.4.2, with only BSTFA and TMCS which served as the first batch. All 13 samples were then analysed by GC-MS, as specified in Section 4.5. The above-mentioned process was repeated for the second batch of MTBSTFA derivatisation.

4.6.2 Spiked synthetic urine sample standards to assess stability differences of the two derivatisation methods

The spiked synthetic urine sample was prepared as described in Section 4.3.2. Two 15 ml Kimax tubes were used, where 1 ml of the spiked synthetic urine sample was added to each tube. The samples were then extracted as delineated in Section 4.4.1. Afterwards, both samples were derivatised as explained in Section 4.4.2 (one tube was derivatised with TMS and the other with tBDMS) and analysed by GC-MS, as specified in Section 4.5.

4.6.3 Comparison of precision of the two derivatisation methods

The ERNDIM sample and the two QC samples as mentioned in Section 4.3.4, were prepared in triplicate for both derivatisation methods, by adding 1 ml of each sample to 15 ml Kimax tubes. An extraction procedure was then carried out as stated in Section 4.4.1. After the extraction was carried out, the samples were derivatised by both TMS and tBDMS respectively, transferred to GC vials as described in Section 4.4.2, and analysed by GC-MS as specified in Section 4.5.

The % CV was calculated from the normalized peak areas (by dividing the area of the compound with the area of the IS). Those values were used to generate a boxplot in order to compare the variation between the two derivatisation methods.

4.6.4 Second-phase separation for contaminant removal/reduction

1 ml of the spiked synthetic urine sample, prepared in Section 4.3.2, was taken and transferred to two 15 ml Kimax tubes respectively. The first sample (tube) served as the control sample and the second sample (tube) was where the second phase separation was to be done. An extraction procedure was then carried out as stated in Section 4.4.1. After the extraction was carried out, the samples were derivatised with only tBDMS as described in Section 4.4.2. The first sample was then transferred to a GC vial containing an insert, and the second sample was transferred to a GC vial without an insert. 100 µl of Hexane and 50 µl of distilled water were added to the second sample. The sample was briefly vortexed and left to stand for a second-phase separation to occur. The top layer of hexane, possibly containing all important organic acids, was collected and transferred to a GC vial with an insert. Both samples were then analysed by GC-MS as specified in Section 4.5.

All experimentation was proceeded by sample preparation, a familiarization period which included in-house training, and competency assessment. Unexpected findings were repeated and confirmed for confirmation purposes.

CHAPTER 5: RESULTS AND DISCUSSION

5.1 Introduction

This study aimed to investigate an alternative derivatising reagent for GC-MS assay to ultimately quantify biological organic acids in urine. This aim was proposed to improve the derivatisation step during organic acid analysis through the comparison of two derivatising reagents. This was done by assessing the chromatograms of all individual and spike synthetic urine samples and their mass spectra (Section 5.2), the derivatisation stability of spiked synthetic urine samples (Section 5.3), the precision of both derivatising methods (Section 5.4) and additional selectivity (second phase separation) on the spiked synthetic urine sample utilizing tBDMS derivatisation (Section 5.5).

5.2 Chromatograms and Mass Spectrum of the individual and spiked synthetic urine samples.

All possible derivatisable TMS/tBDMS groups with their corresponding [M-15] and/or [M-57] groups were explored through their mass spectrum. The mass spectrum of the individual organic acids is displayed in Section 5.2.1, where the remaining data are displayed in Annexure A. Additionally, an extracted ion chromatogram (EIC) was obtained for each of the organic acid standards. This allowed for the number of peaks and the abundance of the peaks on the EIC of TMS derivatives to be compared with tBDMS derivatives. The data gathered for the EIC of all representative organic acids are displayed in Section 5.2.2 in two examples, where the remaining data will be added to Annexure A.

Initially, no fragment ions were found for 4-Hydroxyphenylpyruvic acid and Succinylacetone. Upon further investigation, it became noticeable that these compounds show signs of keto-enol tautomerism, a second or third group of TMS or tBDMS attached to the abovementioned compounds, as seen in Table 5-1.

5.2.1 Mass spectrum of all organic acids

It is vital that derivatisation must be completed, and it is essential that all possible groups that can derivatise on an organic acid compound, must have derivatised. Thus, it was imperative that all possible fragment ions were explored to ensure no other possible [M-15] and [M-57] were missed during the identification process. It is also important to keep in mind that there are organic acid

sub-classes, namely Keto acids, that are subjected to Tautomerism, and can thus form two (or more) derivatives upon derivatisation (Chalmers, 2012).

When the mass spectrum was investigated for the individual standard of 4-Hydroxyphenylpyruvic acid produced a very abundant 3tBDMS [M-57] ion and less abundant 3TMS ions, and only produced one peak for both TMS and tBDMS derivatives. In the spiked synthetic urine sample, 4-Hydroxyphenylpyruvic acid produced 2 peaks for tBDMS, a very prominent 2tBDMS and a less prominent 3tBDMS. On the contrary, no TMS derivatives were found in the spiked synthetic urine sample. Furthermore, Succinylacetone produced 2 peaks for both TMS and tBDMS, 2TMS, 3TMS, 2tBDMS and 3tBDMS, in the individual standard where the abundances of the tBDMS derivatives were far more intense than the TMS derivatives. In both cases mentioned above, the compounds' [M-15] and [M-57] ions were not found when looking for their fully derivatised ions. Upon further investigation, it became noticeable that these two compounds had possibly undergone tautomerism. Since keto acids are prone to form enols, they need to undergo oximation to stabilise. The fact that the tBDMS derivatives produced much more abundant peaks than the TMS derivatives for both compounds mentioned above, makes the possibility of keto acids needing to undergo oximation less required.

As seen in Table 5-1, the number of peaks found in the individual standards vs the spiked synthetic urine sample differ for Aconitic acid, Fumaric acid, Hippuric acid and Succinic acid. A possible explanation regarding the presence or absence of the [M-15] and [M-57] ions in the individual standards vs the spike synthetic urine samples is that there could have been a shift in dervatisation equilibrium between TMS and tBDMS compounds when the spiked synthetic urine sample was used.

Table 5-1: All TMS and tBDMS fragment ions explored.

	1 TMS		2 TMS		3 TMS		1 tBDMS		2 tBDMS		3 tBDMS		
	Derivatised weight g/mol	[M-15]	Derivatised weight g/mol	[M-15]	Derivatised weight g/mol	[M-15]	Derivatised weight g/mol	[M-57]	Derivatised weight g/mol	[M-57]	Derivatised weight g/mol	[M-57]	Number of theoretical derivatistion possibilities
2-Hydroxybutyric acid	176,25	161	248,45	**233**	N/A	N/A	218,34	161	**332,63**	**275**	N/A	N/A	2
*2-Hydroxybutyric acid	176,25	161	**248,45**	**233**	N/A	N/A	218,34	161	**332,63**	**275**	N/A	N/A	
3-Phenyllactic acid	238,26	223	**310,46**	**295**	N/A	N/A	280,35	223	**394,64**	**337**	N/A	N/A	2
4-Hydroxyphenylpyruvic acid	252,24	237	324,45	309	**Tautomerism 396,64**	**381**	294,33	237	408,63	351	**Tautomerism 522,92**	**465**	2
*4-Hydroxyphenylpyruvic acid	252,24	237	324,45	309	Tautomerism 396,65	381	294,33	237	**408,63**	**351**	Tautomerism 522,93	465	
Aconitic acid	246,22	231	381,42	303	**390,62**	**375**	288,31	231	402,6	345	**516,89**	**459**	3
*Aconitic acid	246,22	231	381,42	303	**390,62**	**375**	288,31	231	402,6	345	**516,89**	**459**	
Adipic acid	218,26	203	**290,46**	**275**	N/A	N/A	260,35	203	**374,64**	**317**	N/A	N/A	2
Benzoic acid	**194,24**	**179**	N/A	N/A	N/A	N/A	**236,33**	**179**	N/A	N/A	N/A	N/A	1
Ethylmalonic acid	204,24	189	**276,44**	**261**	N/A	N/A	246,31	**189	**360,62**	**303**	N/A	N/A	2
Fumaric acid	188,21	173	**260,41**	**245**	N/A	N/A	230,3	173	**344,6**	**287**	N/A	N/A	2
*Fumaric acid	188,21	173	**260,41**	**245**	N/A	N/A	**230,3**	**173**	**344,6**	**287**	N/A	N/A	
Hippuric acid	**251,26**	**236**	323,46	308	N/A	N/A	**293,35**	**236**	407,64	350	N/A	N/A	2
*Hippuric acid	**251,26**	**236**	323,46	308	N/A	N/A	**293,35**	**236**	407,64	350	N/A	N/A	
Lactic acid	162,23	**147	**234,43**	**219**	N/A	N/A	204,32	**147	**318,61**	**261**	N/A	N/A	2
Stearic acid	**356,47**	**341**	N/A	N/A	N/A	N/A	**398,56**	**341**	N/A	N/A	N/A	N/A	1
Succinic acid	190,23	175	**262,43**	**247**	N/A	N/A	232,32	175	**346,61**	**289**	N/A	N/A	2
*Succinic acid	190,23	175	**262,43**	**247**	N/A	N/A	**232,32**	**175**	**346,61**	**289**	N/A	N/A	
Succinylacetone	230,26	215	**Tautomerism 302,46**	**287**	Tautomerism 374,66	359	272,35	215	**Tautomersim 386,60**	**329**	Tautomerism 500	443	1
*Succinylacetone	230,26	215	**Tautomerism 302,47**	**287**	Tautomerism 374,67	359	272,35	215	**Tautomersim 386,61**	**329**	Tautomerism 501	443	

*1, 2 and 3 TMS or tBDMS imply the possibility of 1, 2 and 3 TMS or tBDMS group/s that could have attached to a compound. N/A – Not applicable, as the compound doesn't have the possibility for further derivatisation. Tautomerism seen in the table indicates that the specific compound doesn't have the theoretical capability of another derivatisable group, but another group formed possibly due to Tautomerism. The bold values refer to dominant peaks as measured by the abundance of the [M-15] and/or [M-57] ions EIC of all individual organic acids. The normal black values refer to another peak found but in low concentrations. The light grey values refer to the theoretical peaks not found. *Additional rows added to indicate instances where dervatisation differed between individual standards and spiked synthetic urine samples ** [M-15] and/or [M-57] ions that are frequently present in the spectra of most organic acids and are thus inconclusive [M-15] and/or [M-57] ions. These ions are representative of the characteristic ions commonly found in organic acid spectra, as discussed in Sections 2.4.3 and 2.4.4, as they can give identification problems/difficulties and were thus marked with two stars.*

During the search of all the possible derivatisable groups for both TMS and tBDMS derivatives, the spiked synthetic urine samples were examined through samples that were injected multiple times over 24 hours. Furthermore, during the search, the most abundant [M-15] and [M-57] ions that were found were the compounds that had fully derivatised, as seen in Table 5-1 (with the exception of Hippuric acid). These ions were seen as the primary [M-15] and [M-57] ions, as they were found continuously throughout all samples and had the most intense peak abundances in comparison to the other [M-15] and [M-57] ions found. In the case where another derivative of TMS and/or tBDMS were found, their abundance was exceedingly small in comparison to the primary [M-15] and [M-57]. Thus, it is apparent that the abundance of the other possible peaks didn't increase over time, nor did the abundance of the fully derivatised peaks decrease due to partial hydrolysis at least. Hippuric acid's primary [M-15] and [M-57] ions seem to be the 1TMS and 1tBDMS groups instead of the fully derivatised 2TMS and 2tBDMS groups. An explanation could be that Hippuric acid contains a carboxylic acid and an amine group. Amine and secondary amine groups are less favourable to silylation than a carboxylic acid, as mentioned in Section 2.3.

It thus becomes apparent that the sample matrix influences derivatisation efficiency. This has implications if calibrators are to be used in a quantification method. The calibrators will have to be in the same matrix as the samples otherwise, dervatisation efficiency may not be the same.

5.2.2 Mass spectrum of individual organic acids

5.2.2.1 TIC of all organic acids

In Section 2.4.3 and Section 2.4.4, the characteristic fragment ions expected to be found in the mass spectrum of TMS and tBDMS derivatives were outlined per organic acids subclass. The

fully derivatised [M-15] and [M-57] fragment ions were found primarily throughout all samples and were thus used predominantly for further dissection of the mass spectrum of individual organic acids. Apart from their primary [M-15] and [M-57] fragment ions that were found throughout all organic acids, their mass spectrum will be discussed with a focus on the characteristic fragment ions expected to be seen in their mass spectra.

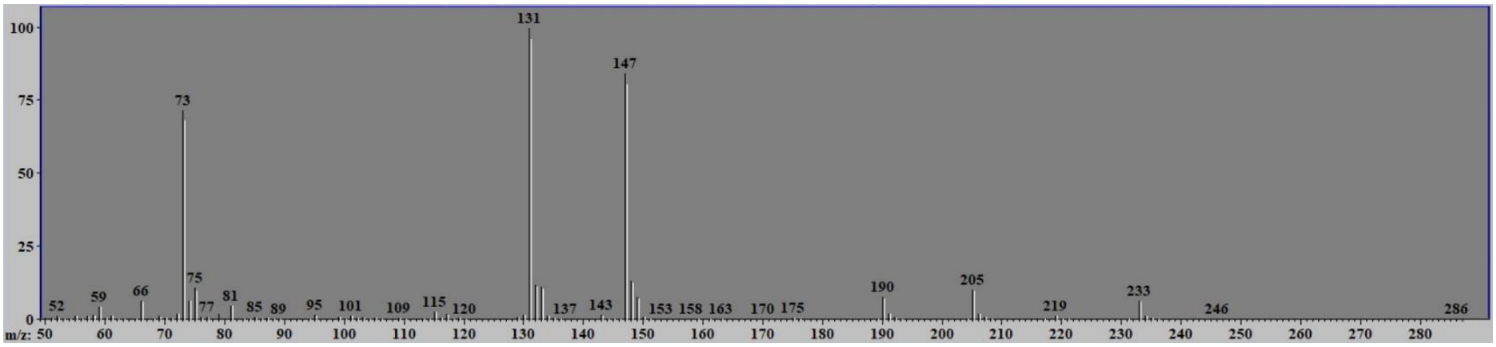


Figure 5-1: MS of the [M-15] ion of the TMS derivatives of 2-Hydroxybutyric acid.

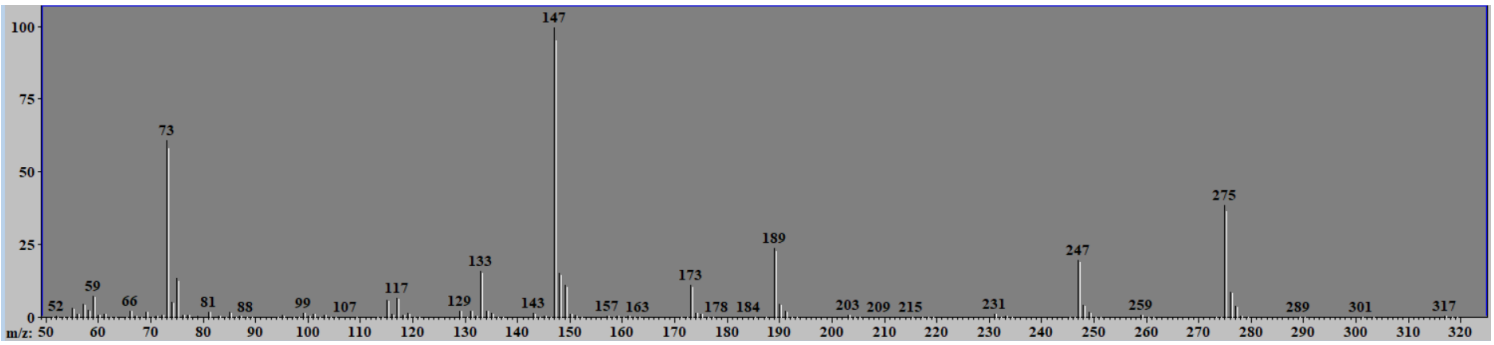


Figure 5-2: MS of the [M-57] ion of the tBDMS derivatives of 2-Hydroxybutyric acid.

Beginning with Stearic acid, belonging to the subclass mono-carboxylic acid, Stearic acid displayed all the expected fragment ions in its MS for its TMS and tBDMS derivative (Figure A.19 and Figure A.20). All Dicarboxylic acids and Tricarboxylic acids produced all expected fragment ions to be seen in their mass spectrum for their TMS derivatives (as seen in Figures; A.5, A.7, A.11, A.13 and A.21). On the contrary, not all tBDMS derivatives produced all expected fragment ions, but it is important to keep in mind that the tBDMS derivatives had a much broader profile of expected ions than the TMS derivatives. All tBDMS derivatives of Dicarboxylic acids and Tricarboxylic acids did however produce a majority of the expected fragment ions throughout,

namely; 73, 75, 115, 133 and 147 m/z with varying intensities which can be seen in Figures A.6, A.8, A12, A.14 and A.22. The TMS derivatives of the Benzoic acids also did not display all expected characteristic fragment ions but did produce 77 m/z and 105 m/z in high intensities throughout (Figures A.9 and A.15). Their tBDMS derivatives did, however, produce all the major expected (77 m/z and 105 m/z) characteristic fragment ions and most of the minor expected characteristic fragment ions 51, 73, 75 and 135 m/z, except for 189 m/z (Figures A.10 and A.16). All TMS derivatives of the Bifunctional compounds (Figures 5-1, A.1 and A.17) produced the expected characteristic fragment ions, where 73 m/z and 147 m/z remained in high intensities and 131 m/z varying in intensities. Furthermore, their tBDMS derivatives (Figures 5-2, A.2 and A.18) produced all major expected characteristic ions with the intensities remaining persistent to some degree, while the minor fragments were not found in all Bifunctional groups. Both the TMS derivatives and tBDMS derivatives of the Keto-Acids (Figures A.3, A.4, A.23 and A.24) produced the expected characteristic fragment ions of 73 m/z and 147 m/z, with Succinylacetone's 147 m/z intensity varying from the tBDMS derivative of 4-Hydroxyphenylpyruvic acid's 147 m/z found. It became apparent that for Succinylacetone, 169 m/z seems to be characteristic of the mass spectrum profile, as it produced an abundant peak in its TMS derivative and an even more abundant peak in its tBDMS derivative.

Taking Figure 5-1 as an example, an argument can be made that the most abundant ion (131 m/z) can be used for quantification purposes rather than 233 m/z (the [M-15] ion) that has a relatively small ion intensity, but 131 m/z is too commonly found in organic acids' mass spectrum profiles, which would increase quantification difficulties. Thus, even though some of the [M-15] and [M-57] ions have lower ion intensities than other ions (of a specific organic acid) in their mass spectra, it is ideal to find a unique high abundant fragment ion, like the [M-15] and [M-57] ions.

Another argument can be made that more samples can simply be injected to increase the intensity of the [M-15] ion for TMS derivatives, but MS has a restriction in its linearity. If a person was to inject 5 times more sample, tBDMS' abundance will also be 5 times more, thus the abundance of TMS and tBDMS will remain the same, as TMS derivative fragments more extensively Because of MS' linearity restriction, the problem can thus not be solved through injecting more sample, as all the ions will also be more abundant because a person is past the linearity of the MS.

5.2.2.2 EIC of all organic acids

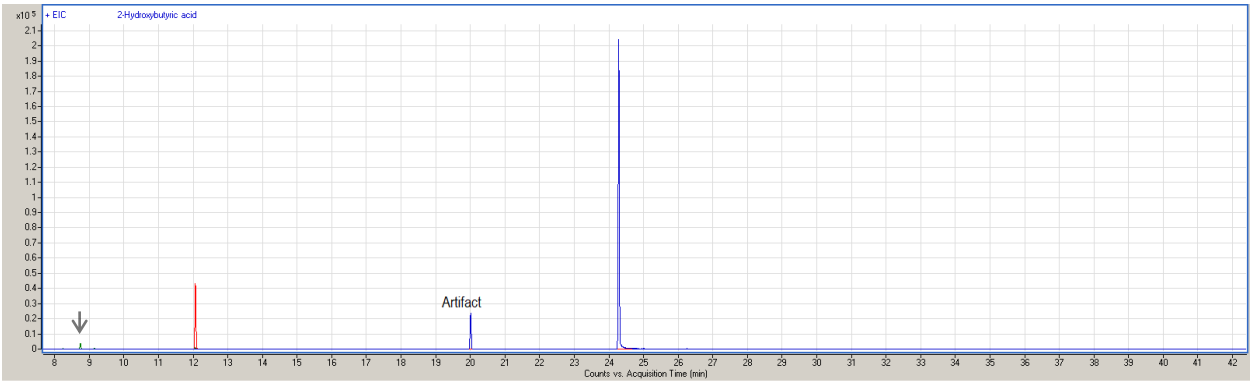


Figure 5-3: EIC of the fragment ion of TMS (red and green line) and tBDMS (blue line) derivatives of 2-Hydroxybutyric acid. The peak abundance of the tBDMS derivative is distinctly higher than the TMS derivative. The green line (indicated by the arrow) = 1TMS and the Red line = 2TMS.

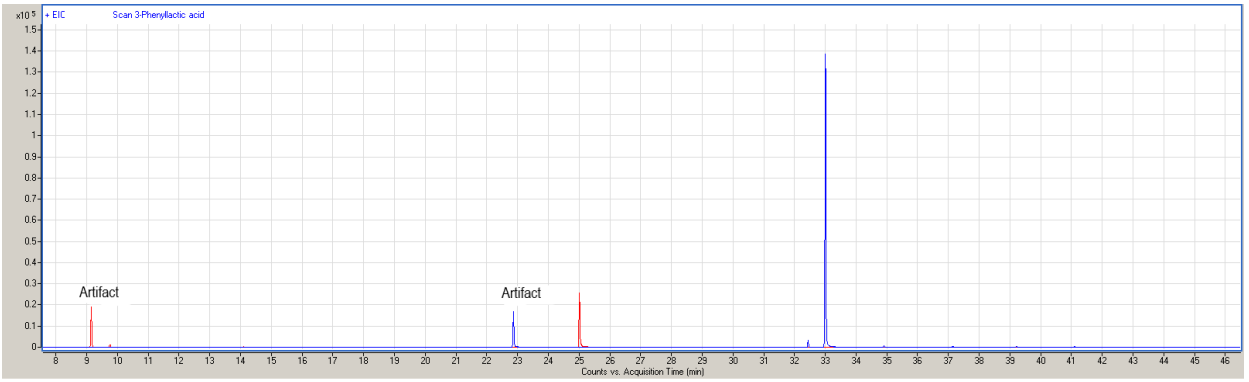


Figure 5-4: EIC of the fragment ions of TMS (red line) and tBDMS (blue line) derivatives of 3-Phenyllactic acid. The peak abundance of the tBDMS derivative is distinctly higher than the TMS derivative.

The abundance of the TMS derivatives ([M-15] ions) for Aconitic acid and Benzoic acid is higher compared to their tBDMS derivatives ([M-57), whereas the tBDMS abundance for 2-Hyroxybutyric acid, 3-Phenyllactic acid, 4-Hydroxyphenylpyruvic acid, Adipic acid, Ethylmalonic, Fumaric acid, Hippuric acid, Lactic acid, Stearic acid, Succinic acid and Succinylacetone's tBDMS derivatives are higher than their TMS derivatives. In the case where the [M-15] ion abundance of the TMS derivatives Aconitic acid and Benzoic acid are higher than their tBDMS derivative [M-57] ion, it is only by a small fraction (Figure B.2 and Figure B.4). On the contrary, the peak abundance of the

tBDMS derivatives of 2-Hyroxybutyric acid, 3-Phenyllactic acid, 4-Hydroxyphenylpyruvic acid, Adipic acid, Ethylmalonic, Fumaric acid, Hippuric acid, Lactic acid, Stearic acid, Succinic acid and Succinylacetone, compared to their TMS derivatives, are distinctly higher (Figure 5-3, 5-4, B.1, B.3, B.5, B.6, B.7, B.8, B.9, B.10 and B.11). The tBDMS derivative for the two organic acids that produces slightly more abundant TMS derivatives, still proves to be quantifiable as the peak abundance is only less abundant by a small fraction.

As mentioned, Ohie *et al.* (2000) reported that, by using TMS derivatives, particular ion fragments have ion intensities that remain small, causing the quantification sensitivity to be restricted by mass chromatography. Furthermore, there was also proclaimed that when using silyl derivatives other than TMS (e.g., tBDMS), the molecular weight will increase and their retention times may shift dramatically (Halket & Zaikin, 2003). However, it can be advantageous when compounds with low molecular weights need to be analysed (Halket & Zaikin, 2003), as the mass will increase significantly.

The results reported in Section 5.2 concur with the above. The use of tBDMS derivatisation increased the molecular weight significantly in comparison to TMS derivatisation, but as seen in the results in Section 5.2, TMS derivatives [M-15] ion intensities were in most cases very low, which would make quantification more difficult. These results implicate that using MTBSTFA as a derivatisation reagent (to produce tBDMS derivatives), organic acids will likely yield better quantifiable results than compared to using BSTFA and TMCS (to produce TMS derivatives) as a derivatisation reagent.

Furthermore, as stated by Hintikka *et al.* (2008) and Nguyen *et al.* (2013), as well as concluded in this study in Section 5.2 and Annexure A, tBDMS derivatives produce a more intense fragment ion of [M-57] compared to TMS derivatives' fragment ion of [M-15].

tBDMS groups more frequently formed 2 fragment ions than TMS derivatives (Figures 5-3, 5-4, B.1, B.2, B.3, B.4, B.5, B.6, B.7, B.8, B.9, B.10 and Table 5-2). This could be due to Steric hindrance of the tBDMS derivatives, as their molecular masses are higher than TMS derivatives, making steric hindrance more of a possibility pertaining to tBDMS groups (Schummer *et al.*, 2009). Regardless of the possible steric hindrance occurrence, the majority of tBDMS derivatives remained stable and had thus no influence over the data attained. Even though tBDMS derivatives more frequently form two fragment ions, the second ions that were produced were in low concentrations, and therefore tBDMS derivatisation will still be feasible for quantification.

Succinylacetone is a supplementary example of a compound that may be misidentified when present in small amounts (low concentrations) in urine or may disappear in alkaline solutions (Kałużna-Czaplińska, 2011). Succinylacetone is a vital marker of Tyrosinemia type I, which makes the identification of this compound substantial. Using MTBSTFA as a derivatising reagent increases the likelihood of identifying organic acids like Succinylacetone, as mentioned above since MTBSTFA has the ability to increase the molecular mass significantly for organic acids with low molecular masses, specifically as some organic acids are present in small amounts (low concentrations). As seen in Figure B.11, it becomes evident that tBDMS produces distinctly more superior peaks for quantification, thus diminishing the possibility of misidentifying the compound.

Table 5-2: TMS and tBDMS derivatives producing 0, 1 or 2 peaks in the individual standard samples and the spiked synthetic urine samples.

	SU TMS No Peaks	Individual Standards TMS No Peaks	SU tBDMS No Peaks	Individual Standards tBDMS No Peaks
2-Hydroxybutyric acid	1	2	1	1
3-Phenyllactic acid	1	1	1	1
4-Hydroxyphenylpyruvic acid	0	1	1	2
Aconitic acid	1	1	2	1
Adipic acid	1	1	1	1
Benzoic acid	1	1	1	1
Ethylmalonic acid	1	1	1	1
Fumaric acid	1	1	2	1
Hippuric acid	2	1	1	1
Lactic acid	1	1	1	1
Stearic acid	1	1	1	1
Succinic acid	1	1	2	1
Succinylacetone	1	2	1	2

SU: Spiked synthetic urine.

5.3 Derivatisation stability of spiked synthetic urine sample profiles

The stability for both derivatisation methods was evaluated, as depicted in Section 5.3.1 where the EIC of all organic acids' fragment ions is displayed on one graph. In Section 5.3.2, the stability of each sample will be displayed and compared making use of line graphs.

5.3.1 The EIC of all representative organic acids in the spike synthetic urine sample to assess their on-column stability.

The EIC of all TMS and tBDMS derivatives are displayed in Figure 5-5 and Figure 5-6 respectively, portraying all the representative organic acids that were injected multiple times over 24 hours. These multiple injections are displayed simultaneously for each organic acid.

Please note that 4-Hydroxyphenylpyruvic acid was not found while derivatised with TMS, thus the only discussion pertaining to the stability of 4-Hydroxyohenylpyrivic acid will be of 4-Hydroxyohenylpyruvic acid derivatised with tBDMS.

Figure 5-5 and Figure 5-6 illustrate that the tBDMS derivative of 2-Hydroxybutyric acid, Hippuric acid and Succinylacetone produced exceedingly better symmetrical peak shapes in comparison to their TMS derivative, albeit peak tailing is visible in the tBDMS derivative of Hippuric acid. Moreover, 3-Phenyllactic acid, 4-Hydroxyphenylpyruvic acid, Aconitic acid, Adipic acid, Benzoic acid, Ethylmalonic acid, Fumaric acid, Lactic acid, Stearic acid and Succinic acid all produced symmetrical peaks with no extensive peak tailing for both TMS and tBDMS derivatives. Stearic acid does however have slight peak tailing that occurs.

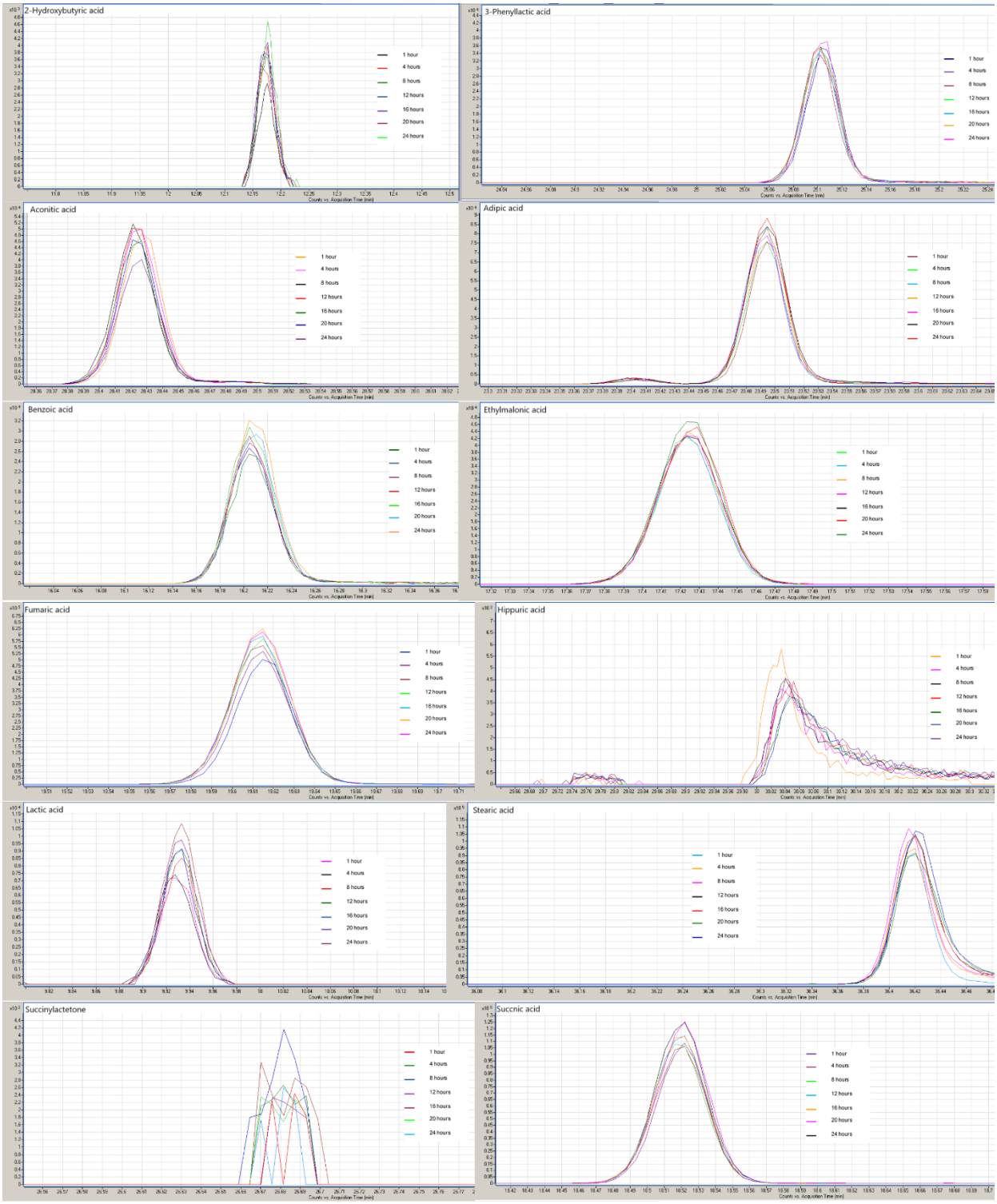


Figure 5-5: EIC of all TMS derivatives with multiple sequential injections of all organic acids.

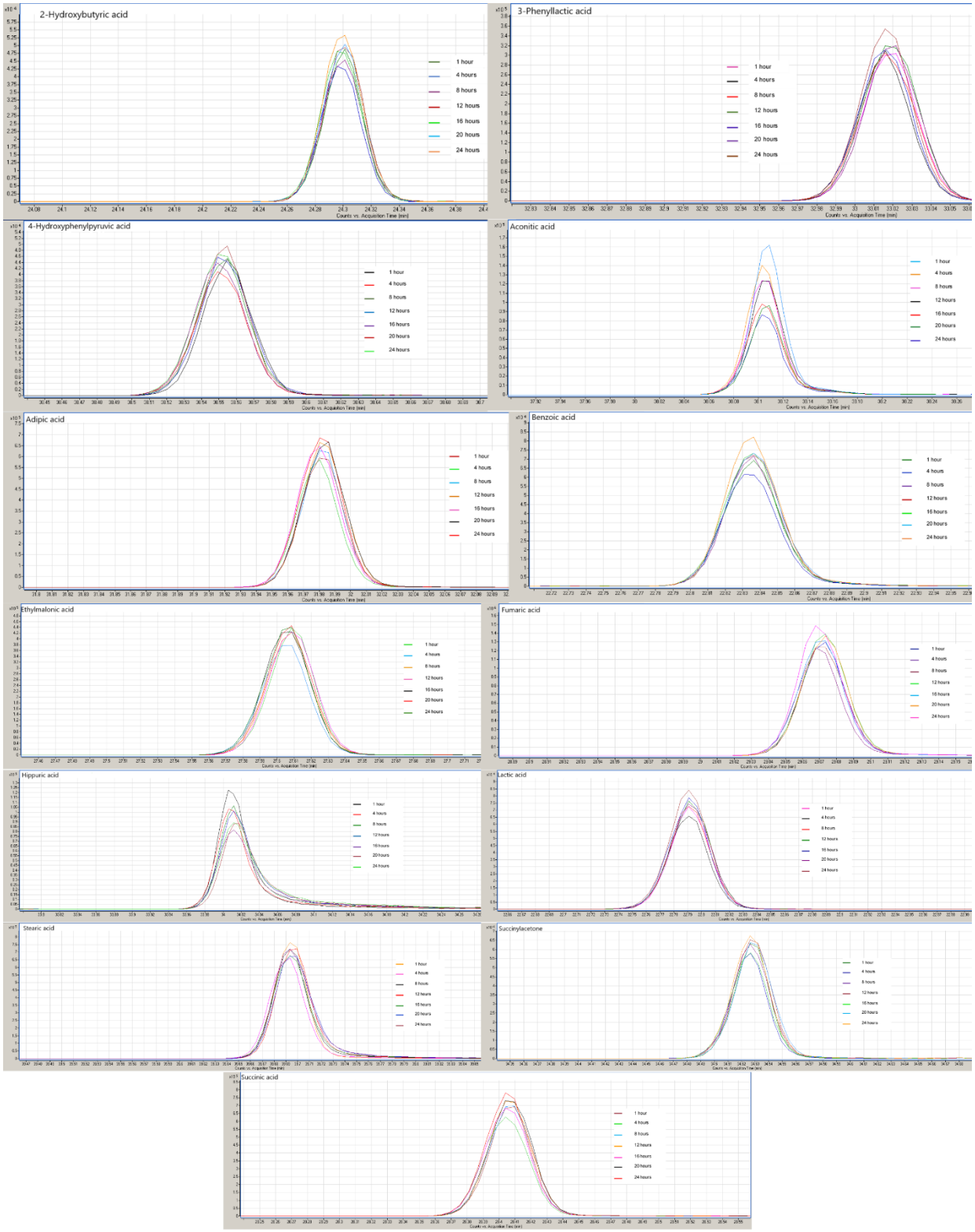


Figure 5-6: EIC of all tBDMS derivatives with multiple sequential injections of all organic acids.

5.3.2 Quantitative evaluation of the stability of organic acid profiles.

The abundance of the [M-15] and [M-57] ions of all organic acids, for both TMS and tBDMS derivatisation methods, were extracted by generating a line graph for all representative organic acids. Furthermore, a line graph overlay of all representative organic acids investigated displaying the percentage change in area over multiple injections is also presented.

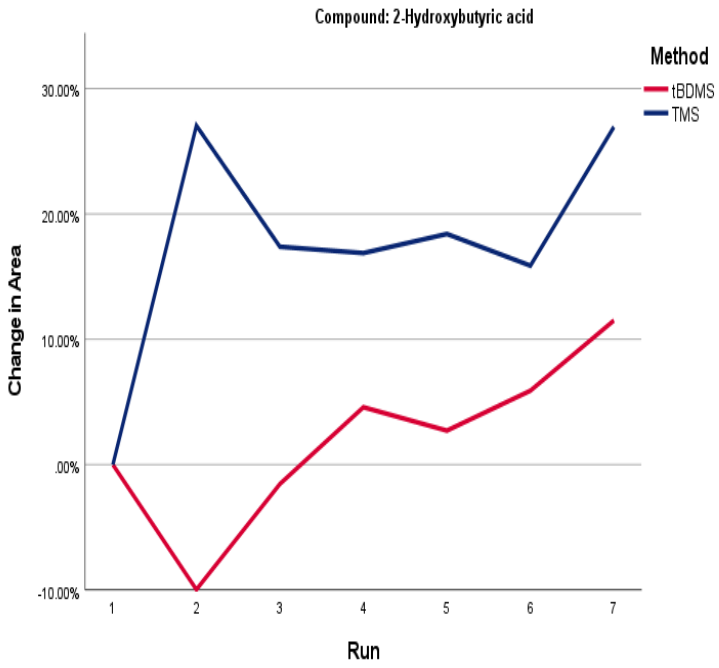


Figure 5-7: Line graph depicting the percentage change in area by run for TMS (blue) and tBDMS (red) of 2-Hydroxybutyric acid over multiple sequential injections. The tBDMS derivative of 2-Hydroxybutyric acid demonstrates better stability than the TMS derivative.

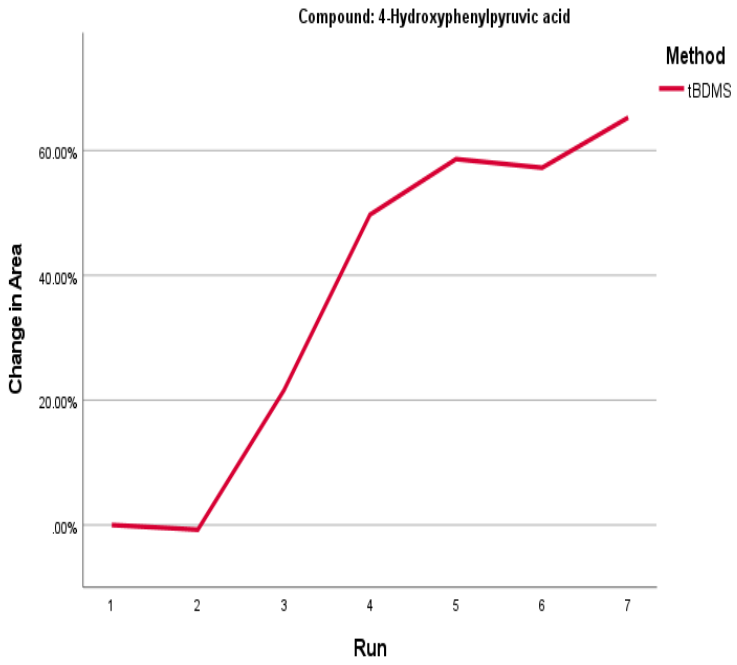


Figure 5-8: Line graph depicting the change in Area by run and by method for 4-Hydroxyphenylpyruvic acid. Derivatisation by tBDMS appears to be unstable over 24 hours.

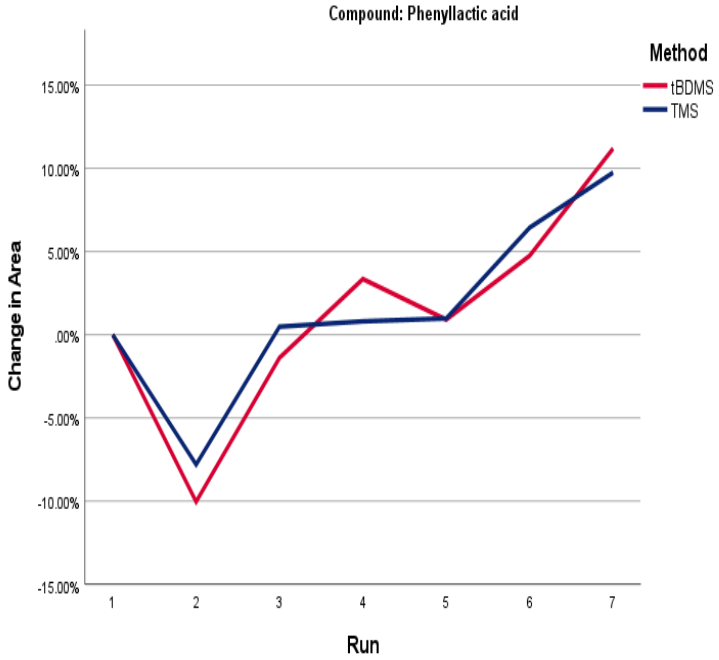


Figure 5-9: A line graph depicting the change in area by run and by method for 3-Phenyllactic acid. Both tBDMS and TMS derivatives demonstrate comparable stability.

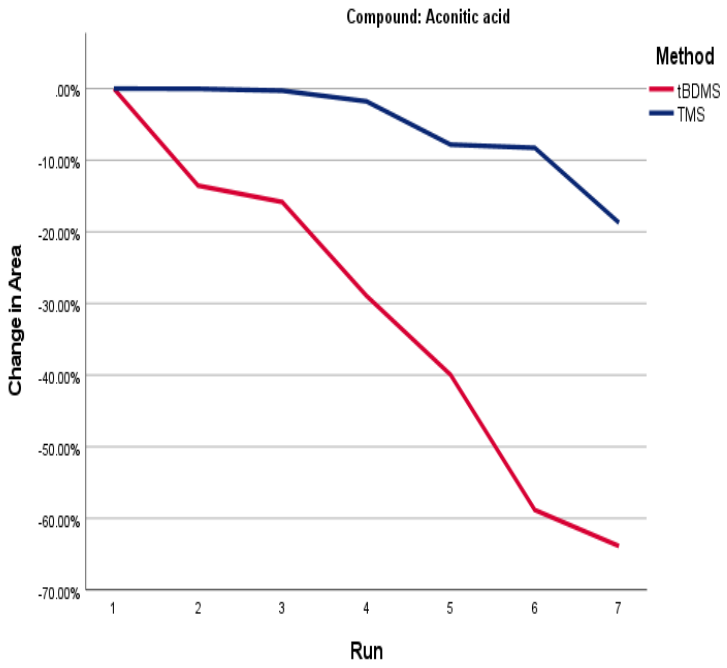


Figure 5-10: A line graph depicting the change in Area by run and by method for Aconitic acid. The TMS derivative of Acontic acid demonstrates better stability than the tBDMS derivative.

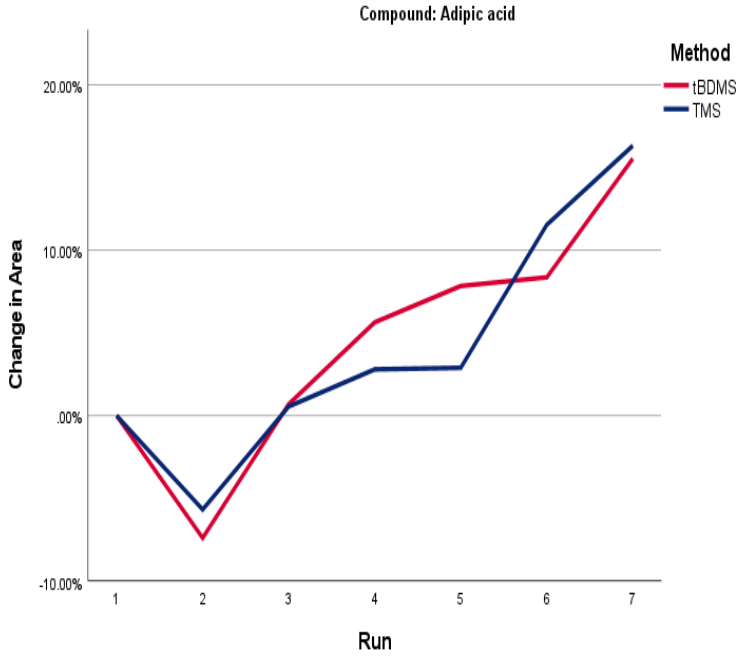


Figure 5-11: A line graph depicting the change in Area by run and by method for Adipic acid. Both tBDMS and TMS derivatives demonstrate comparable stability.

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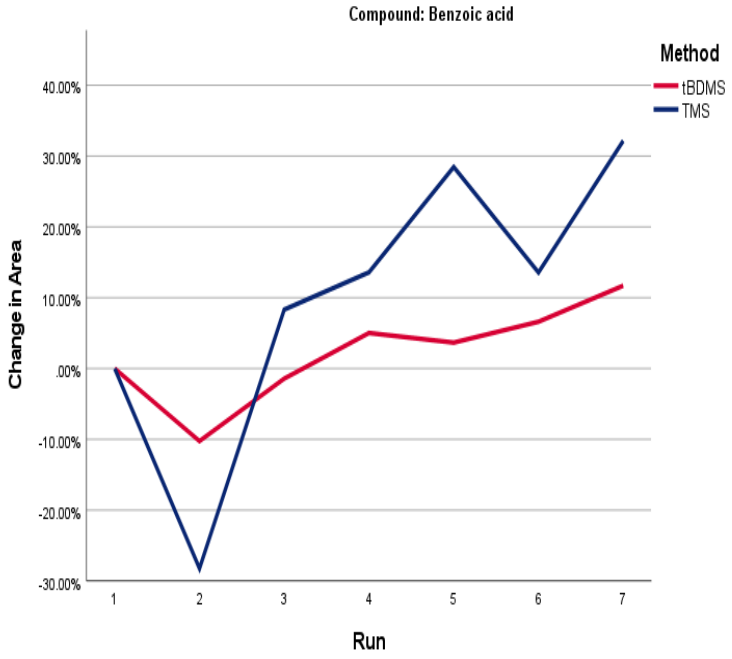

Figure 5-12: A line graph depicting the change in Area by run and by method for Benzoic acid. The tBDMS derivative of Benzoic acid demonstrates better stability than the TMS derivative.

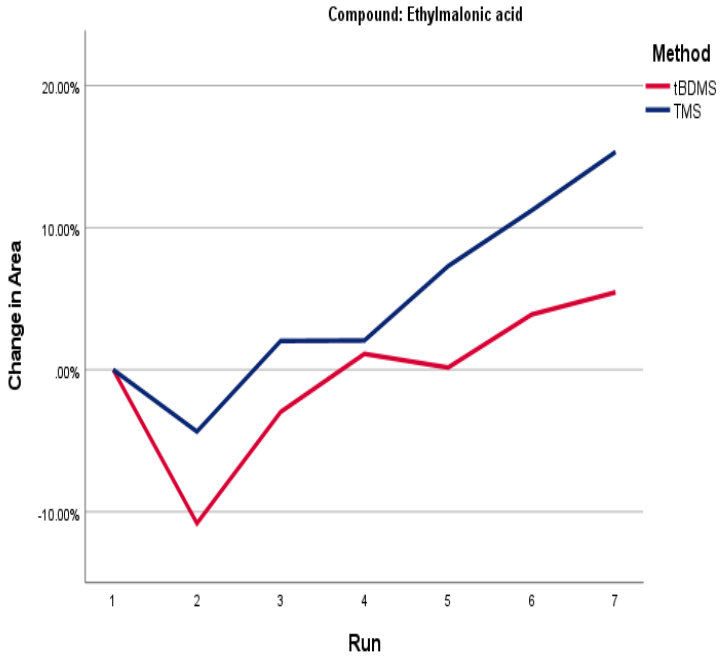

Figure 5-13: A line graph depicting the change in Area by run and by method for Ethylmalonic

acid. The tBDMS derivative of Ethylmalonic acid demonstrates better stability than the TMS derivative.

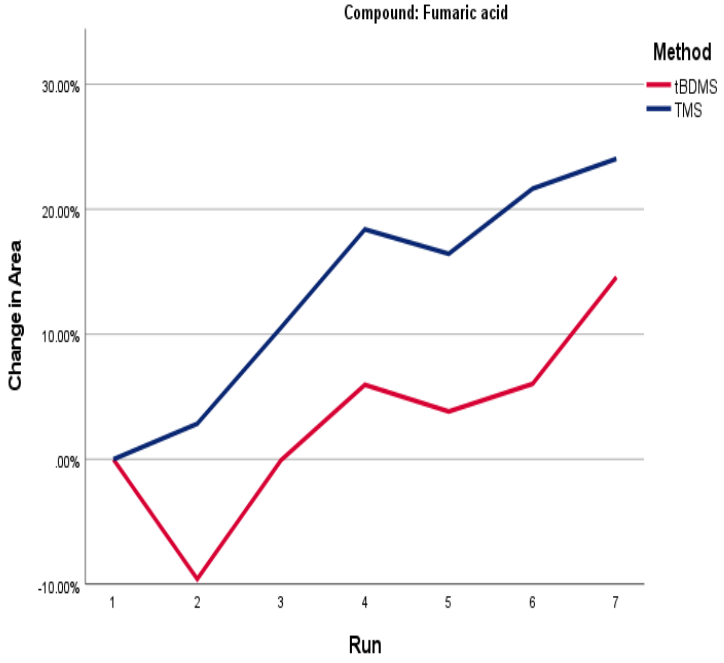

Figure 5-14: A line graph depicting the change in Area by run and by method for Fumaric acid. The tBDMS derivative of Fumaric acid demonstrates better stability than the TMS derivative.

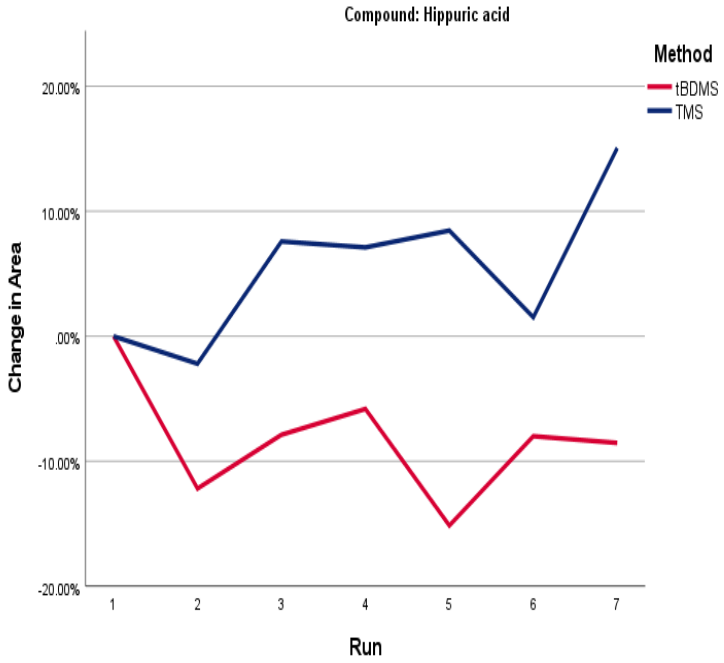

Figure 5-15: A line graph depicting the change in Area by run and by method for Hippuric acid. The tBDMS derivative of Hippuric acid demonstrates better stability than the TMS derivative.

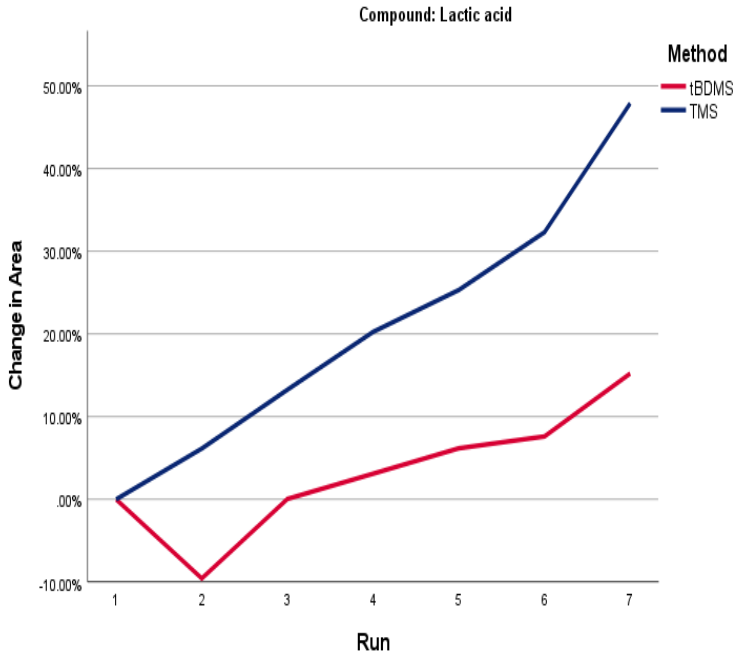

Figure 5-16: A line graph depicting the change in Area by run and by method for Lactic acid. The tBDMS derivative of Lactic acid demonstrates better stability than the TMS derivative.

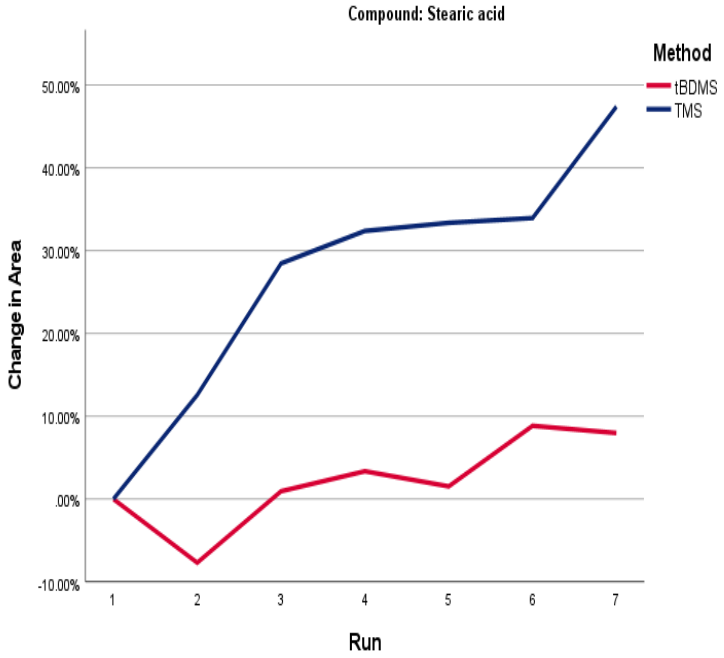

Figure 5-17: A line graph depicting the change in Area by run and by method for Stearic

acid. The tBDMS derivative of Stearic acid demonstrates better stability than the TMS derivative.

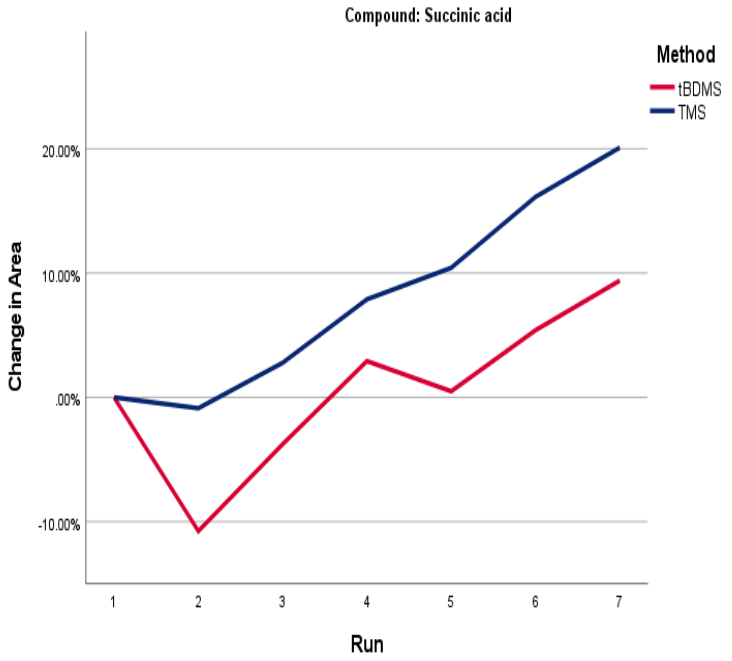

Figure 5-18: A line graph depicting the change in Area by run and by method for Succinic acid. The tBDMS derivative of Succinic acid demonstrates better stability than the TMS derivative.

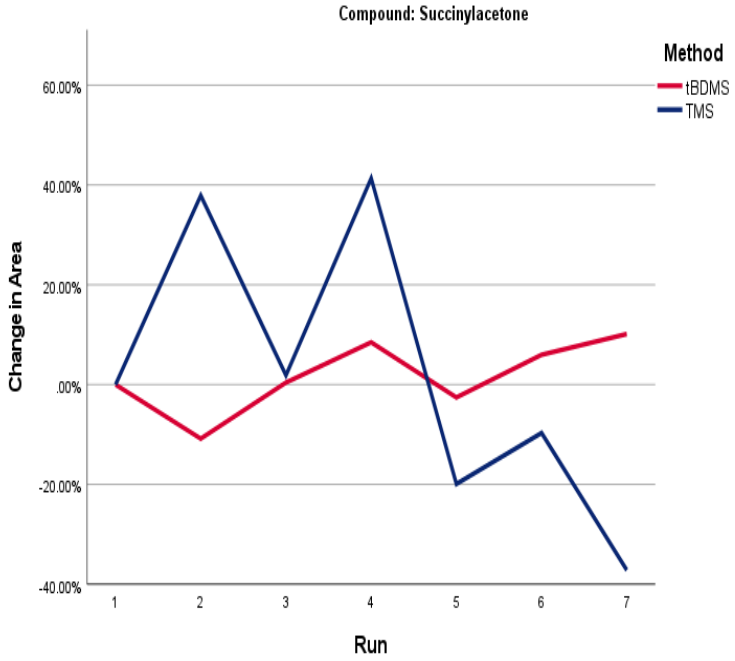

Figure 5-19: A line graph depicting the change in Area by run and by method for Succinylacetone acid. The tBDMS derivative of Succinylacetone acid demonstrates better stability than the TMS derivative.

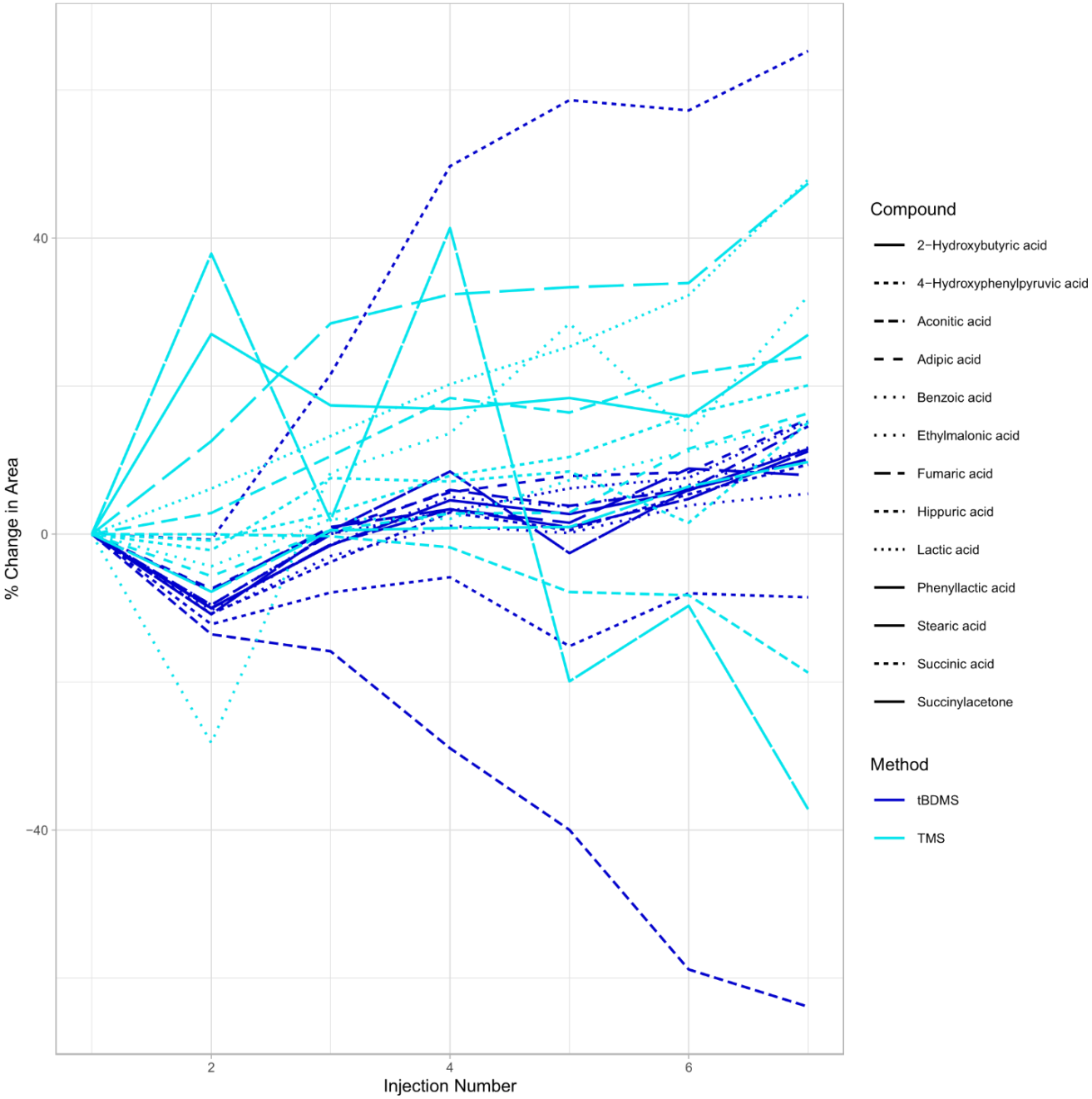

Figure 5-20: Line graph depicting the percentage change in area over multiple sequential injections of all organic acids. tBDMS displays greater stability over a 24-hr period when compared to TMS. Two exceptions to this are Aconitic acid and 4-Hydroxyphenylpyrivic acid. Note

that 4-hydroxyphenylpyruvic acid could however not be detected after TMS dervatisation. For clarity, the stability is again presented in Figure 5-21 after the exclusion of the two tBDMS outliers.

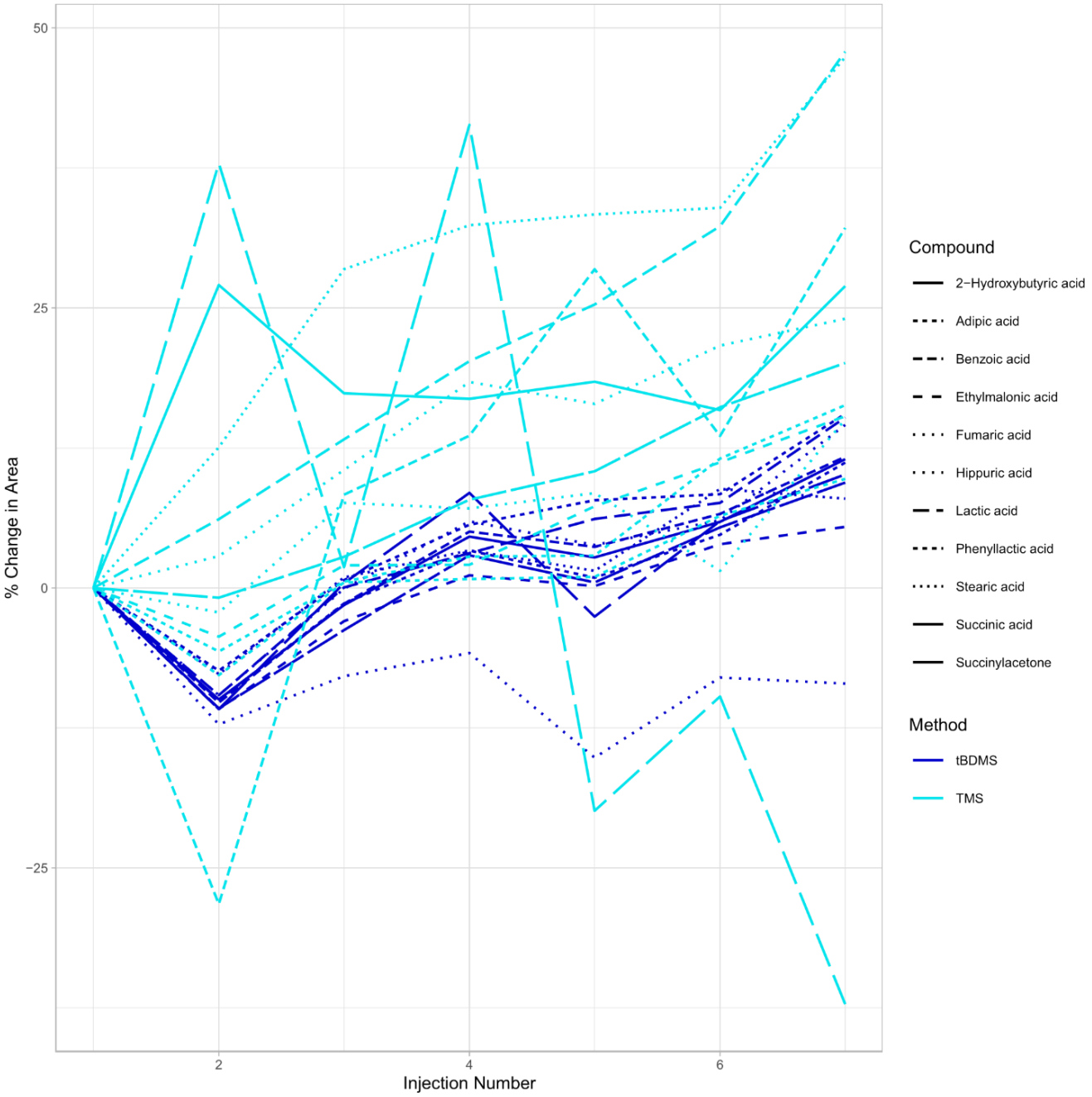

Figure 5-21: Line graph depicting the percentage change in area over multiple sequential injections for all organic acids, excluding compounds showing extreme instability (4-Hydroxyphenylpyruvic acid & Aconitic acid).

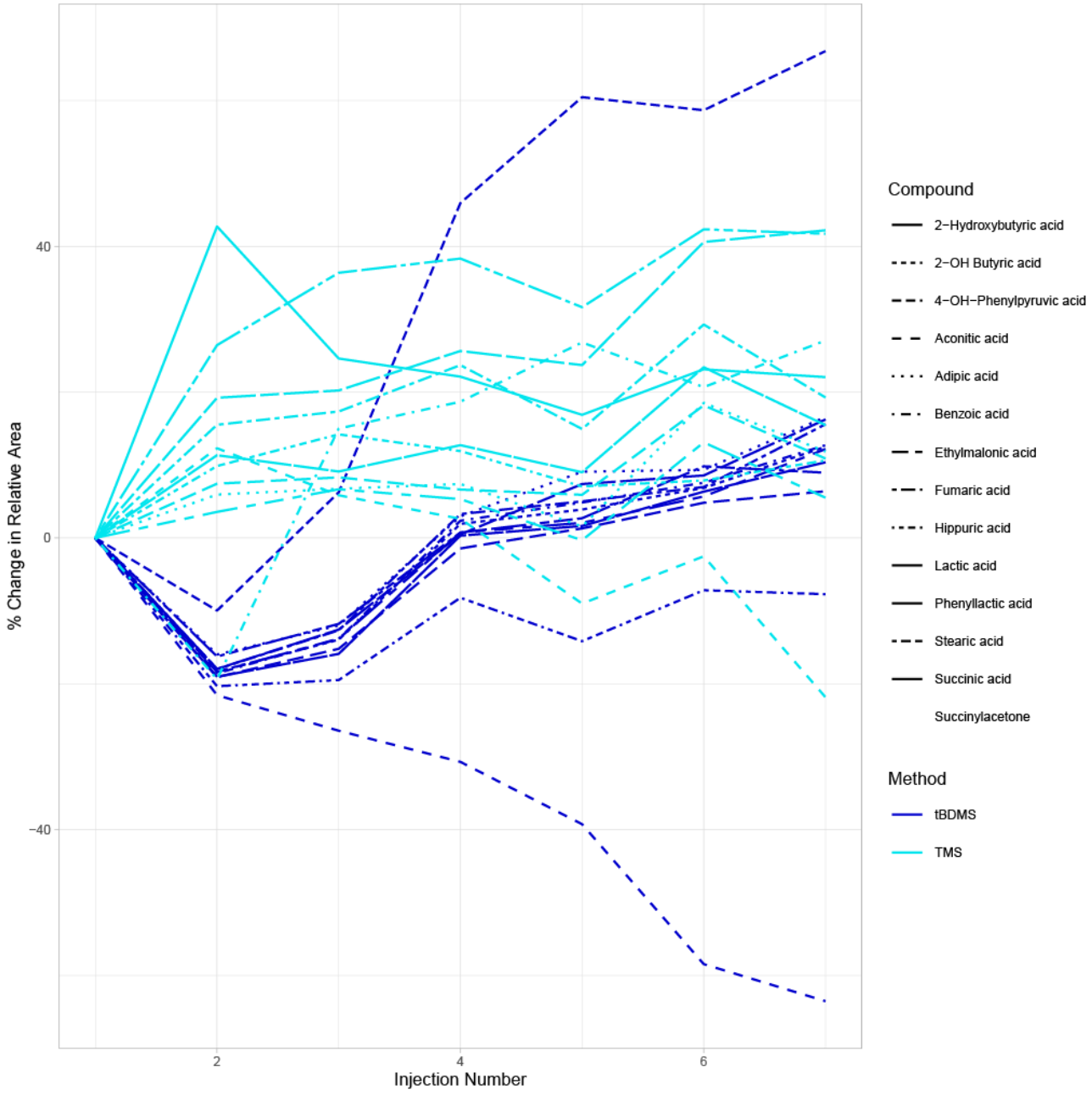

Figure 5-22: Line graph depicting the relative percentage change in area after normalization to the internal standard over multiple sequential injections of all organic acids.

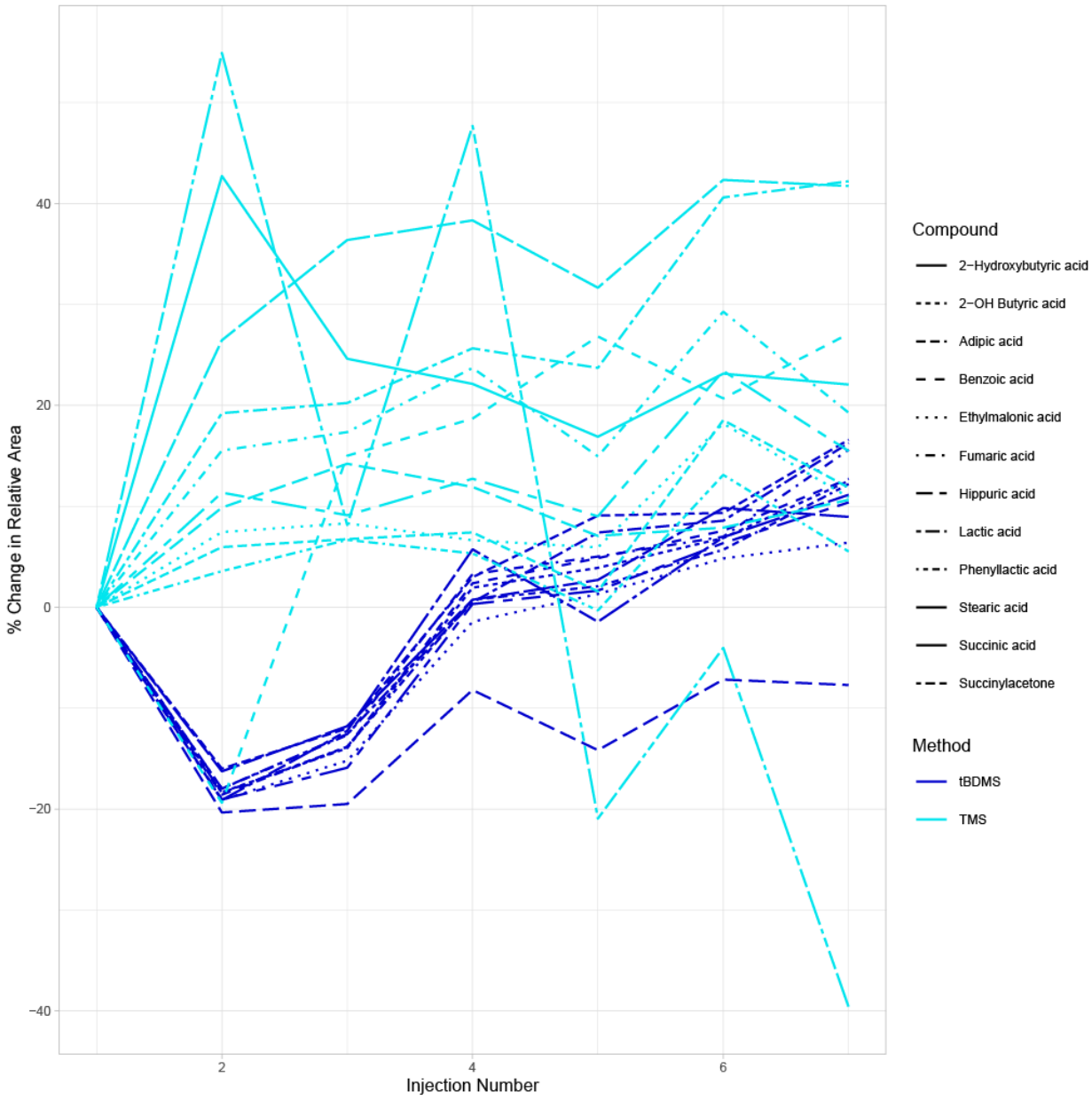

Figure 5-23: Line graph depicting the relative percentage change in the area after normalization to the internal standard over multiple sequential injections for all organic acids, excluding compounds showing extreme instability (4-Hydroxyphenylpyruvic acid & Aconitic acid).

2-Hydroxybutyric acid, Benzoic acid, Ethylmalonic acid, Fumaric acid, Hippuric acid, Lactic acid, Stearic acid, Succinic acid and Succinylacetone (nine of the thirteen representative organic acids) showed that their tBDMS derivatives are more stable over time, as seen throughout Figure 5-5 to Figure 5-21. Moreover, 3-Phenyllactic acid and Adipic acid (two of the representative

organic acids) showed the same degree of stability, as seen in Figure D.1 and Figure D.3. Aconitic acid is the only organic acid where its TMS derivative is slightly more stable, 4-Hydroxyphenylpyrivic acid only detected its tBDMS derivative, which showed to be relatively unstable over 24 hours but would still be viable for identification purposes.

Figure 5-9 clearly illustrates that there are two outliers of tBDMS derivatives, namely 4-Hydroxyphenylpyruvic acid and Aconitic acid. This is in contrast to the TMS derivative which appears to be completely unstable. A speculation however can be that the tBDMS derivatives will tend to be more stable, as formerly described in Chapter 2. As previously established in this section, Aconitic acid's TMS derivative seems slightly more stable than its tBDMS derivatives, thus making Aconitic acid the other outlier seen in Figure 5-20. When taking the above-mentioned outliers out of account, it becomes evident in Figure 5-21 that the tBDMS derivatives of the organic acids seem more stable than their TMS derivatives over 24 hours, as the percentage change in the area of the tBDMS derivatives is much more stable, presenting less variability in comparison with the TMS derivatives.

Figures 5-22 and 5-23 illustrate the relative percentage change in abundance for both TMS and tBDMS derivatives after internal standard normalization. Internal standard normalization was not initially undertaken to see that its own instability would have clouded the results. It is recognised, however, that the changes observed in Figures 5-16 and 5-17 are not necessarily due to instability. The results could for example also be explained by changes in ionization efficiency. Such changes can be partially corrected by normalization to the internal standard. However, as seen in Figure 5-22 and Figure 5-23 internal standard normalisation had a minimal effect on the observed differences between TMS and tBDMS dervatisation. Thus, it can be said with more certainty that tBDMS derivatives present much less variation and are much more stable overall than TMS derivatives.

5.4 Precision comparison of both derivatisation methods.

In Figure 5-24 and Table 5-3, the variation of TMS derivatives can be seen in comparison to tBDMS derivatives. TMS CV%, excluding the extreme outliers, ranges from 25%-47%, 6%-49% and 5%-24% for ERNDIM, QC1 and QC 2 samples respectively. TMS has extreme outliers that range from 52%-57%, 50%-84% and 64%-92% for ERNIDM, QC1 and QC2 respectively. tBDMS CV%, excluding extreme outliers, ranges from 0,5%-38%, 1%-33% and 1%-27% for ERNDIM, QC1 and QC 2 samples respectively, with outliers only in QC1 (133,3%) and QC 2 (64,88%). With that being said, the overall CV% for the tBDMS derivatives is much better than the TMS

derivatives. The difference in variation seen between TMS derivatives and tBDMS derivatives can be due to the fact that tBDMS derivatives have much more abundant [M-57] ions in comparison to the TMS derivatives' [M-15] ions.

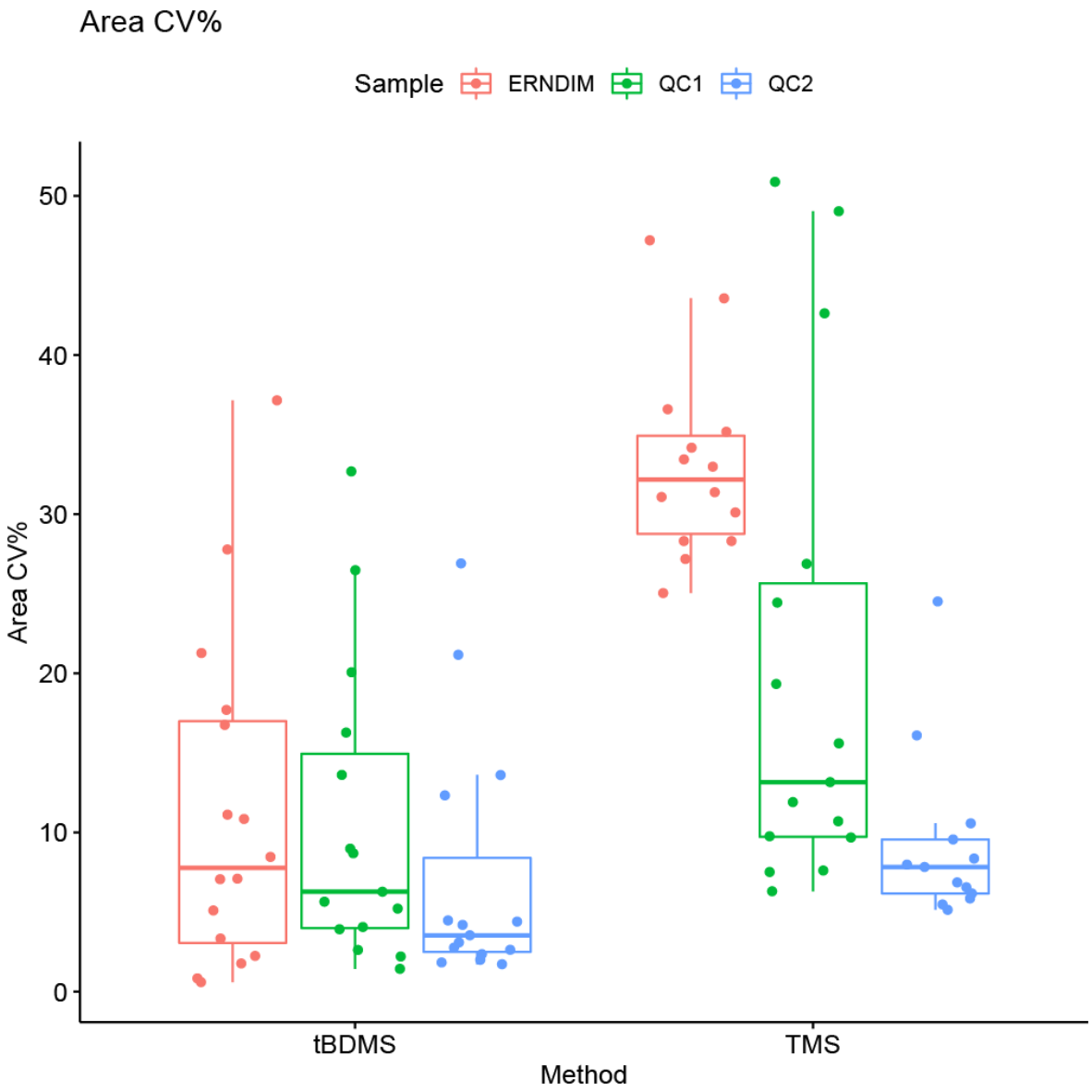

Figure 5-24: Boxplot representing the measurement of precision between the two derivatisation methods for ERNDIM and QC samples, with the extreme outliers excluded. The extreme outlier values can be found in Table 5-5.

Table 5-3: Relative concentrations of the organic acids in the ERNDIM and QC samples

	TMS			tBDMS		
Compound	ERNDIM (CV %)	QC1	QC2	ERNDIM	QC1	QC2
2-Hydroxyglutaric acid	0,0339 (31,08)	0,0098 (9,68)	0,0808 (8,36)	0,0392 (0,59)	0,0064 (16,27)	0,0969 (1,72)
3-Hydroxy-3-Methylglutaric acid	N/A	0,0197 (9,76)	0,1272 (24,51)	N/A	0,0001 (26,48)	0,0019 (21,16)
3-Hydroxyisovaleric acid	0,0178 (56,90)	0,0048 (24,44)	0,0313 (7,98)	0,0688 (5,10)	0,0151 (5,21)	0,1619 (3,09)
3-Methylglutaconic acid	0,0150 (35,18)	N/A	N/A	0,0274 (27,78)	N/A	N/A
3-Methylglutaric acid	0,0309 (30,11)	0,0049 (13,16)	0,0373 (7,83)	0,0419 (7,06)	0,0090 (2,61)	0,0510 (3,53)
4-Hydroxybutyric acid	0,0047 (43,56)	0,0021 (24,21)	0,0198 (6,86)	0,0053 (8,46)	0,0169 (133,30)	0,0238 (26,90)
Adipic acid	0,0705 (34,17)	0,0145 (6,30)	0,1770 (6,17)	0,0937 (3,33)	0,0197 (5,65)	0,2283 (4,47)
Ethylmalonic acid	0,0444 (28,31)	0,0093 (42,62)	0,1005 (83,53)	0,0007 (11,12)	0,0145 (4,06)	0,1636 (2,35)
Fumaric acid	0,0419 (32,98)	0,0054 (15,60)	0,1261 (6,55)	0,0539 (7,09)	0,0074 (3,92)	0,1696 (1,83)
Glutaric acid	0,0293 (31,38)	0,0081 (50,88)	0,0572 (91,87)	0,0520 (26,32)	0,0160 (2,20)	0,1792 (4,19)
Glycolic acid	0,0168 (52,64)	0,0027 (26,88)	0,0024 (16,09)	0,0227 (10,84)	0,0045 (20,06)	0,0040 (13,61)
Malic acid	N/A	0,0006 (19,33)	0,0005 (64,67)	N/A	0,0005 (32,68)	0,0003 (64,88)
Methylmalonic acid	0,1572 (25,04)	0,0097 (10,70)	0,1213 (5,85)	0,1673 (37,15)	0,0168 (6,27)	0,0464 (2,76)
Pyroglutamic acid	0,0291 (36,59)	0,0078 (84,52)	0,0523 (10,57)	0,0444 (17,70)	0,0149 (8,98)	0,0621 (2,63)
Sebacic acid	0,0778 (33,44)	0,0215 (7,51)	0,1175 (5,14)	0,0979 (1,76)	0,0200 (13,61)	0,1520 (4,40)
Suberic acid	0,1278 (28,31)	N/A	N/A	0,1627 (2,24)	N/A	N/A
Tiglylglycine	0,0109 (47,21)	0,0054 (11,90)	0,0177 (9,56)	0,0018 (16,75)	0,0148 (8,69)	0,0150(12,33)

Calculation of the relative concentrations: Area of compound/Area of IS. N/A: Compounds were not in that particular matrix and thus no CV% calculation was needed. The highlighted values represent extreme outliers.

5.5 Second phase separation of spiked synthetic urine sample profiles (tBDMS derivatisation).

A TIC (Total Ion Chromatogram) of spiked synthetic urine sample with and without a second liquid-liquid extraction step, was extracted and is displayed in Figures 5-25 and 5-26.

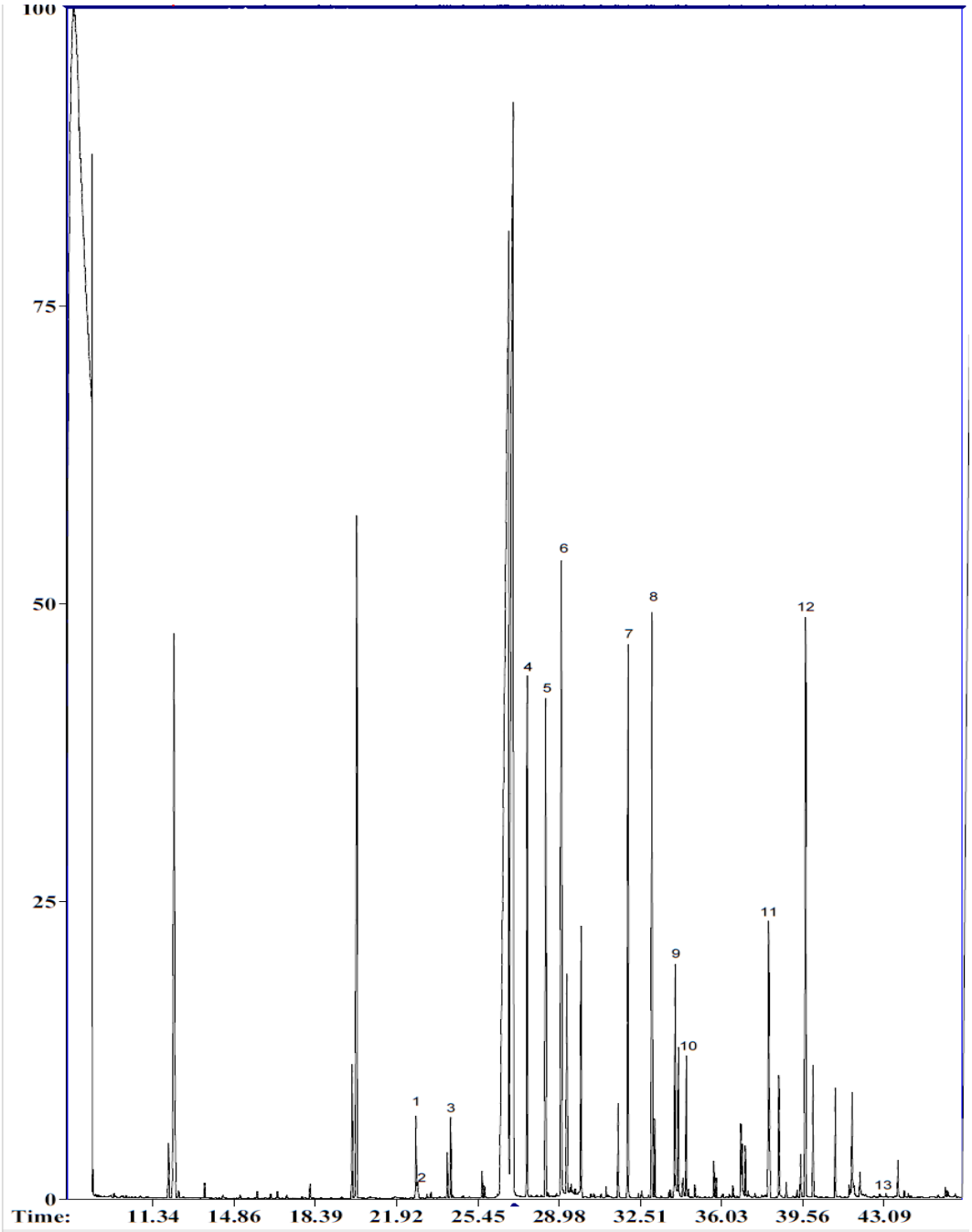

Figure 5-25: TIC of tBDMS control sample. All representative organic acid compounds were found and the specific compounds can be found in Table 5-3.

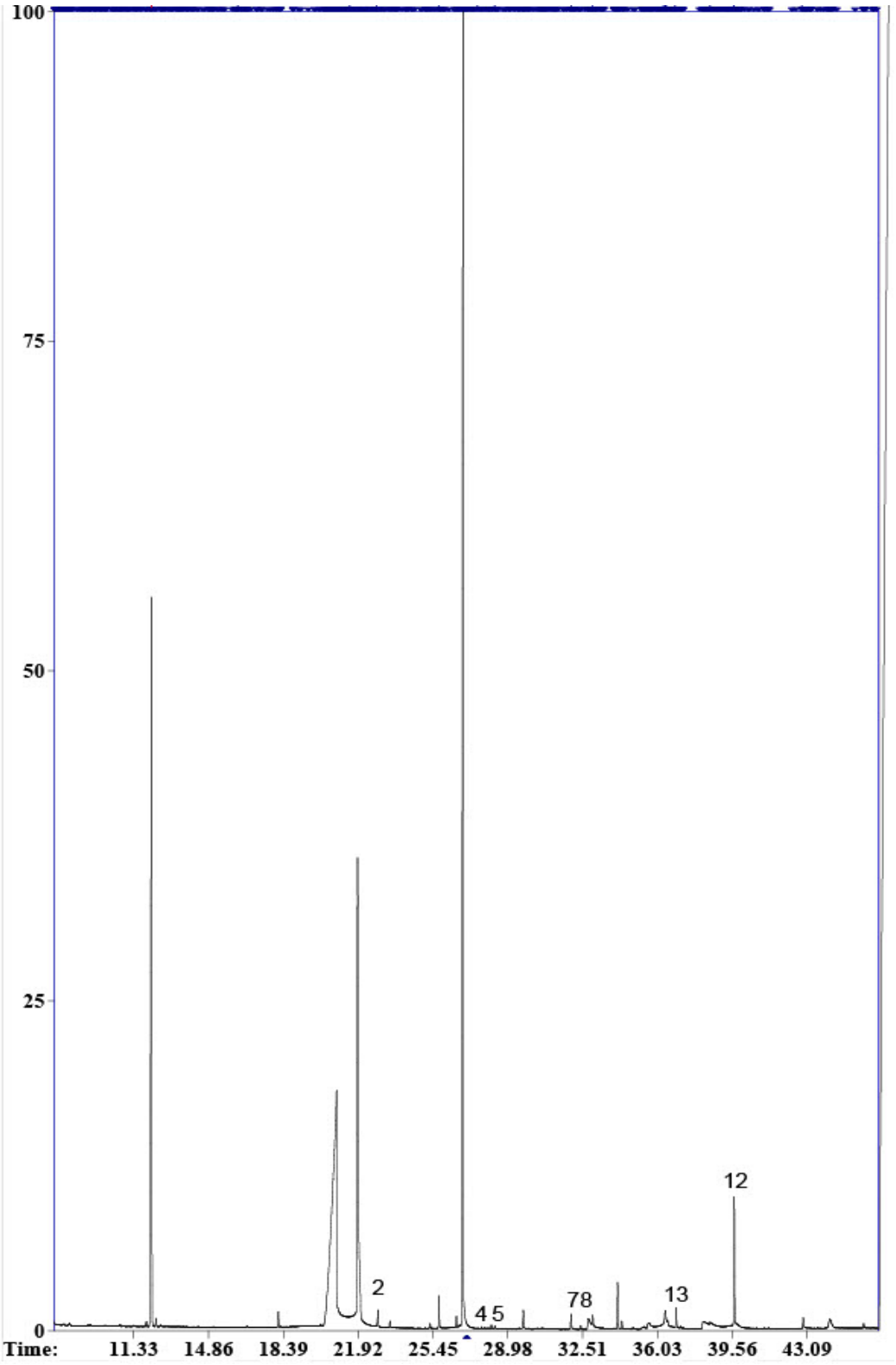

Figure 5-26: TIC of second phase separation with tBDMS sample. 53,8% of the representative organic acid compounds were found. *4-Hydroxyphenylpyruvic acid's 2tBDMS was found in this*

sample in contrast to the control sample where 4-Hydroxyphenylpyruvic acid's 3tBDMS was found.

Table 5-4 Organic acids reference guide for compounds found and not found in Figure 5-25 and Figure 5-26.

Lactic acid	1
Benzoic acid	2
2-Hydroxybutyric acid	3
Ethylmalonic acid	4
Succinic acid	5
Fumaric acid	6
Adipic acid	7
3-Phenyllactic acid	8
Hippuric acid	9
Succinylacetone	10
Aconitic acid	11
Stearic acid	12
4-Hydroxyphenylpyruvic acid	13

Figure 5-25 clearly illustrates that all the representative organic acid compounds were detected, as this sample was used as the control. Figure 5-26 illustrates that only 53,3% of the representative organic acid compounds were detected. Although so many compounds were lost during second phase separation and the abundances are low, there is much less background noise in the spectrum. The data suggest that hexane was perhaps lacking sufficient polarity to extract more polar derivatives. When looking at the abundances with and without second phase nonpolar organic acids, like stearic acid, extracted better than polar compounds, suggesting that a different solvent might have to be used, one that is less polar than Hexane to facilitate more peaks to be produced.

CHAPTER 6: CONCLUSIONS AND FUTURE RECOMMENDATIONS

The need to analyse increasingly smaller amounts (compounds in low concentrations) of compounds has grown exponentially, making it paramount to extend the range of detectability of the compounds in question. Through the process of derivatisation, the detectability of compounds is increased, which enhances the sensitivity of the method. Derivatisation is a technique that generally requires a reaction between the analyte(s) in question and a specific derivatisation reagent. There are numerous derivatisation reagents available for use, however, only TMS and tBDMS derivatisation reagents were used for this study. The addition of a TMS functional group to the compound, also referred to as Trimethylsilylation, is generally used as a derivatisation reagent in GC analyses. Information regarding the chemical structure of compounds can be observed through the mass spectra of TMS derivatives. Additionally, particular ion fragments have ion intensities that remain small, causing the quantification sensitivity to be restricted by MS. High intensity, high [M-15] and/or [M-57] fragment mass ions are needed for quantification, as that particular ion suggests that a compound is stable for quantification. TMS derivatives tend to produce low-intensity, low-fragment mass ions and are thus less stable for quantification. The derivatising reagent tBDMS has advantages over agents that generate TMS derivatives in that tBDMS has higher reactivity and generates moisture-stable derivatives with larger [M-57] ions that confer higher detection sensitivity by MS. Having an appropriate derivatising procedure and reagent should produce the required chemical modification of the compound(s) (thoroughly described in Chapter 2, where both reagents met the required chemical modification necessary for derivatisation to take place), be reproducible, non-hazardous and efficient. Comparisons of cost, variation and availability between tBDMS and TMS derivatisation will be discussed below, along with the individual chromatograms of organic acid standards, derivatisation stability of spiked synthetic urine sample profiles, second phase separation of spiked synthetic urine sample profiles (tBDMS derivatisation), and precision using ERNDIM and QC samples.

In total, five separate analyses were conducted for each of the objectives listed in Chapter 3, where all the representative organic acids were injected and analysed by a GC-MS. For each analysis that was done, all the representative organic acids' tBDMS derivatives were found and detected. In contrast, 4-Hydroxyphenylpyruvic acid's TMS derivative was not found in the second analysis, making this a contributing factor for MTBSTFA derivatisation being more reproducible in this study. Both these derivatisation reagents are hazardous, although safe for laboratory use, as seen when purchased from Sigma Aldrich (Merck). During the purchase of BSTFA + 1% TMCSA and MTBSTFA, from Sigma Aldrich (Merck), it was noticeable that for the same amount

of reagent, MTBSTFA is up to three times more expensive than BSTFA + 1%TMCSA, and both reagents are readily available for purchase.

Even though tBDMS derivatives elute later than TMS derivatives for the same compound, it can still be assured that tBDMS derivatisation is, in addition to larger compounds, better for the derivatisation of smaller compounds. This broadens the possibilities for more compounds to be analysed and identified, where in contrast TMS derivatisation wasn't able to be analysed. Furthermore, tBDMS derivatisation extends the range of detectability of the representative organic acids and could extend the range of detectability for other organic acid compounds. As mentioned in Section 2.4, tBDMS derivatives are stable under humid conditions, thus expanding the possibilities for other analysis (e.g., second phase separation), unlike TMS derivatives which are not stable under humid conditions limiting the use of TMS derivatisation. Moreover, it would be beneficial to assess if increasing the temperature at faster intervals could ultimately elute the compounds faster, and thus address tBDMS derivative retention times.

tBDMS derivatisation is likely to be more proficient in peak identification than TMS derivatisation, as tBDMS derivatives have higher molecular masses than TMS derivatives. Furthermore, although tBDMS derivatives had a higher frequency of producing 2 peaks than TMS derivatives, tBDMS derivatives had much more abundant peaks (even if TMS derivatives only produced 1 peak) and were much more stable. This allows for identification to be more achievable, which will ultimately make the misidentification of organic acids, and possibly other commonly misidentified compounds as well, less frequent. From all the data gathered in this study, it becomes apparent that the majority of tBDMS derivatives have much more intense peak abundances than their corresponding TMS derivatives. Additionally, tBDMS derivatisation could be explored for a GC-MS/MS electron ionisation method as opposed to a chemical ionisation method, as a very abundant [M-57] ion was seen throughout all samples and compounds. When doing MS/MS, one product ion is usually selected for quantification (usually also an abundant high fragment mass ion) while others may serve as qualifiers. These qualifier ions ideally also need to be seen to ensure that the product ion being measured does belong to the precursor ion and thus the compound being measured. Having better knowledge of characteristic ions of organic acids, and in particular characteristic ions of particular classes of organic acids, will aid researchers in selecting the best qualifier ions. Additionally, the data in Chapter 5 suggest that the matrix influences the dervatisation efficiency. Thus, when a targeted method for organic acid analysis is being developed, it should be taken into account that the calibrators should be in an appropriate matrix. This will ensure similar dervatisation efficiencies between the calibrators and the samples.

Furthermore, tBDMS derivatisation could benefit those compounds needing oximation, to stabilise, e.g., Keto acids, as tBDMS could identify these types of compounds without first needing to undergo oximation. As seen in the data (Chapter 5) 4-Hydroxyphenylpyruvic acid and Succinylacetone are Keto acids that tend to be unstable before oximation. In both cases, the tBDMS derivatives were found and an abundant [M-57] was observed, thus certainly more quantifiable.

A good visual summary of the overall difference between the stability of TMS and tBDMS derivatives can be seen in Figures 5-16 through 5-19, where it shows that tBDMS derivatives are predominantly more stable than TMS derivatives. The above declaration also provides empirical evidence in support of Orata (2012), who proposed that tBDMS derivatives can be ten thousand times more stable than TMS derivatives. This will allow, if necessary, for samples that were derivatised with MTBSTFA to endure longer hours of analyses, larger analytical batches, and will still produce more accurate and more quantifiable results than samples that were derivatised with BSTFA +1% TMCS. Overall tBDMS have better precision and less variation compared to TMS derivatives.

The following characteristics are important when selecting a derivatisation reagent, as explained in Chapter 2. The majority of complete derivatives must be produced from the reagent. In the process of derivatisation, there should be no structural change or rearrangements of the compounds. Additionally, there must be no contribution to sample loss. Ultimately, a derivative must be stable with regard to time (Orata, 2012). As seen from the above-mentioned declaration and all the data gathered and discussed in Chapters 5 and 6, a conclusion can be made that tBDMS (MTBSTFA) derivatisation appears to be the more efficient and the predominant derivatisation reagent to use for organic acid analysis, as anticipated.

Although the second phase separation of tBDMS derivatives was not successful in this study, the second phase separation procedure should perhaps be investigated with a more polar solvent. Hexane might have lacked sufficient polarity to extract more polar derivatives in order be to use as a solvent. In addition, incorrect amounts of solvent or water may have been used. The procedure was not optimized and deserves further investigation seeing that it could significantly improve extraction selectivity and minimise contamination of the analytical instrument.

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ANNEXURE A: INDIVIDUAL CHROMATOGRAMS AND MASS SPECTRUM OF ALL ORGANIC ACID STANDARDS.

A.1 MS of Individual organic acids

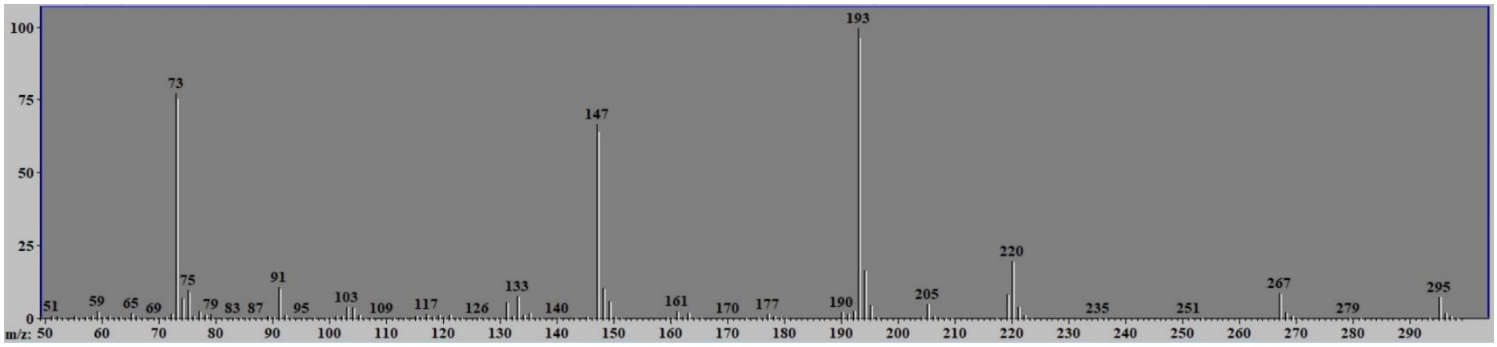

Figure A.1. MS of the TMS derivatives of 3-Phenyllactic acid.

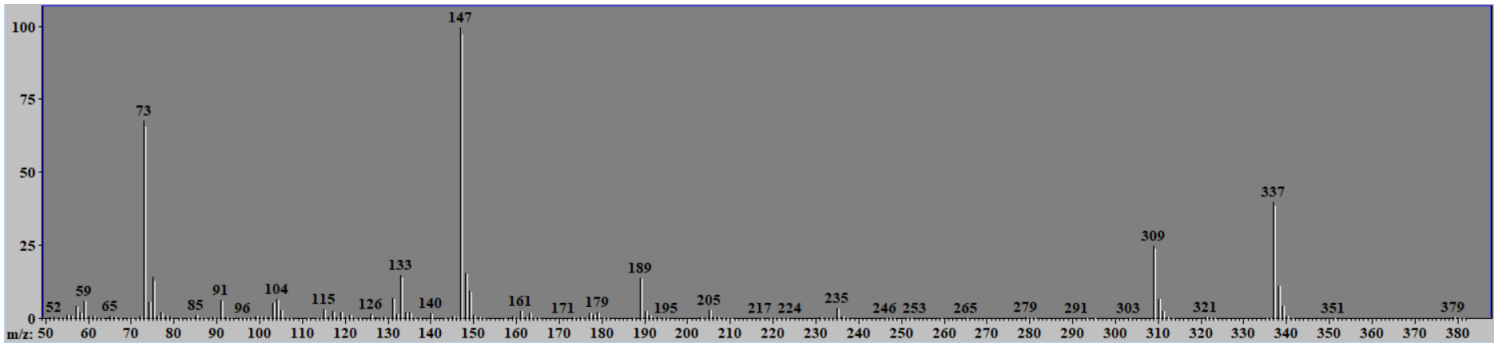

Figure A.2. MS of the tBDMS derivative 3-Phenyllactic acid.

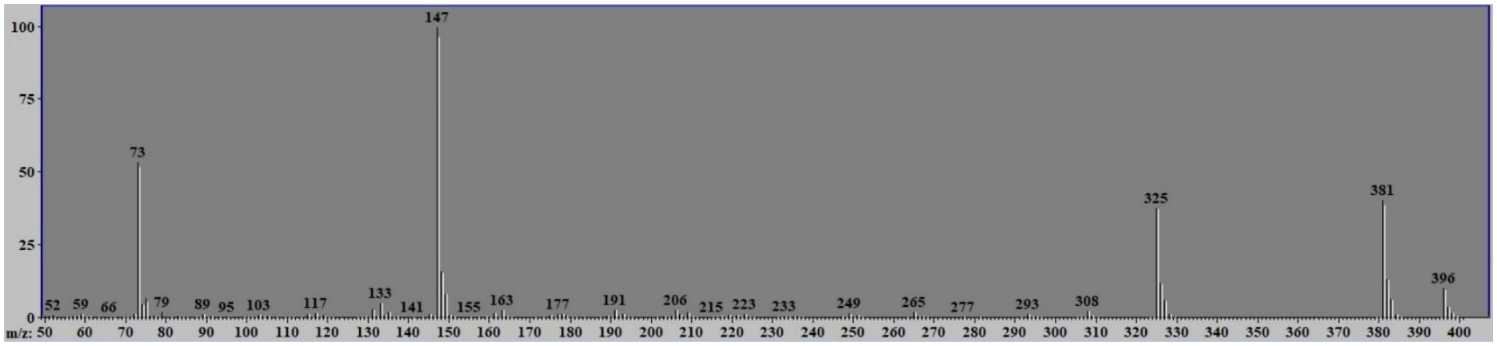

Figure A.3. MS of the TMS derivative 4-Hydroxyphenylpyruvic acid.

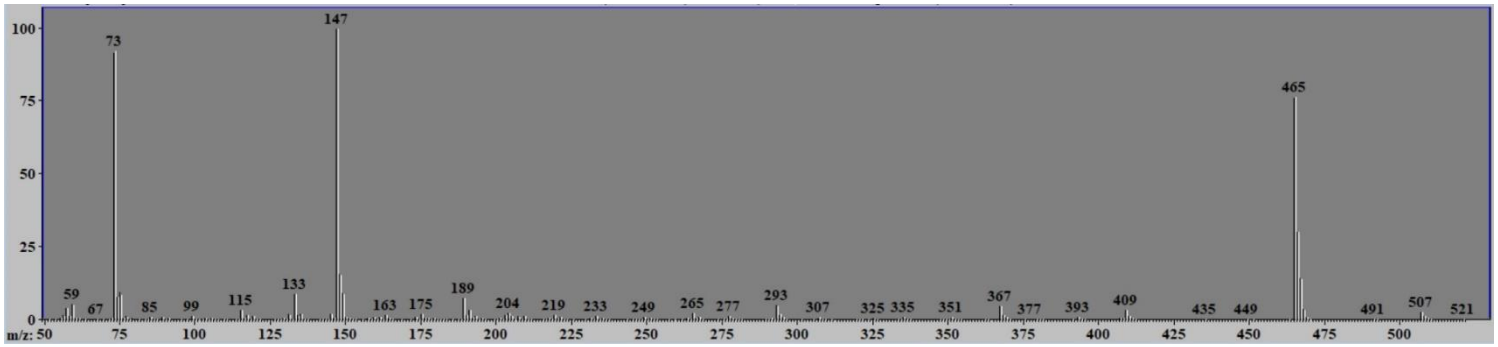

Figure A.4. MS the tBDMS derivative 4-Hydroxyphenylpyruvic acid.

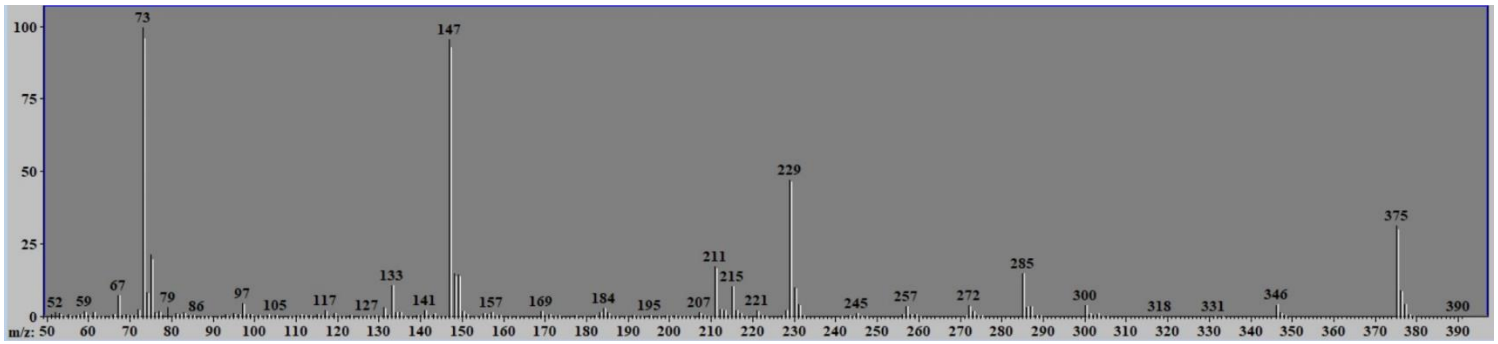

Figure A.5. MS of TMS derivative Aconitic acid.

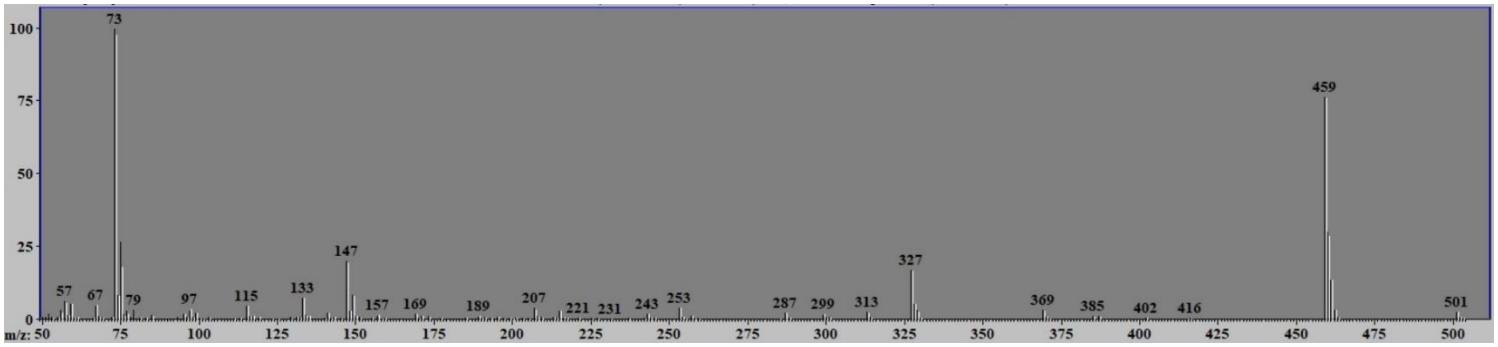

Figure A.6. MS of the tBDMS derivative Aconitic acid.

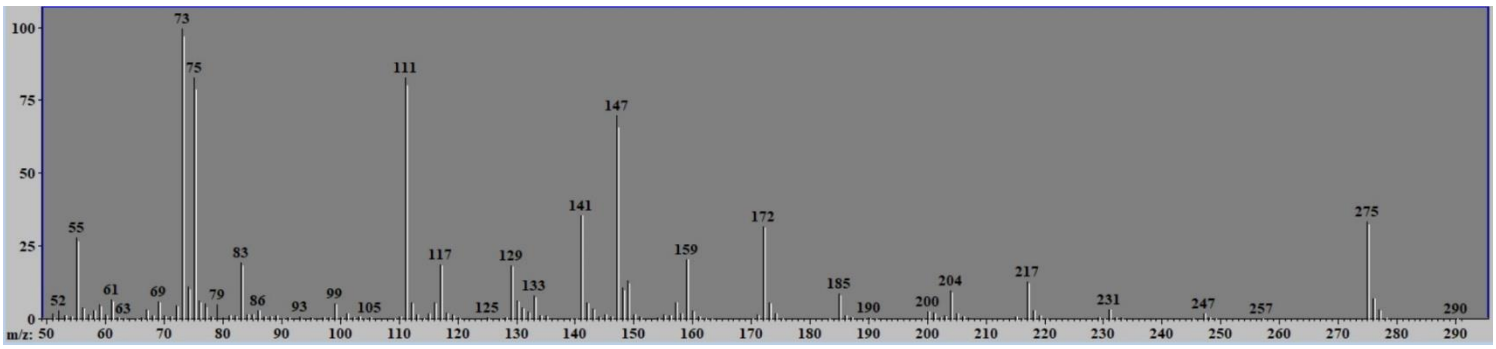

Figure A.7. MS of the TMS derivative Adipic acid.

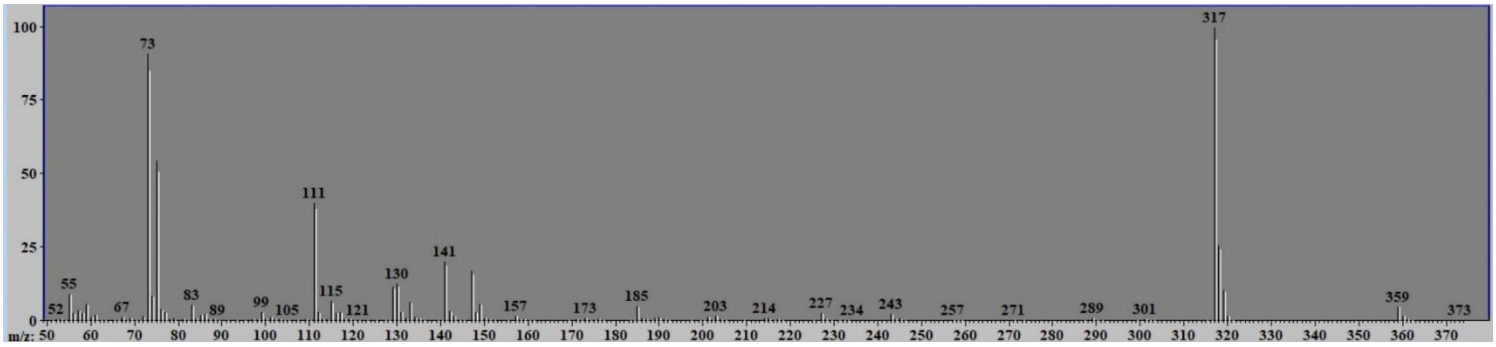

Figure A.8. MS of the tBDMS derivative Adipic acid.

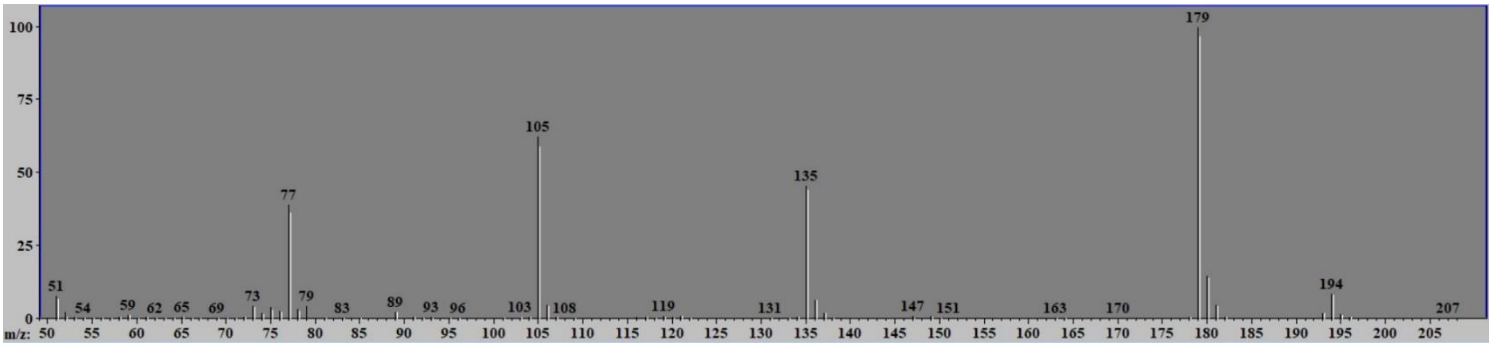

Figure A.9. MS of the TMS derivative Benzoic acid.

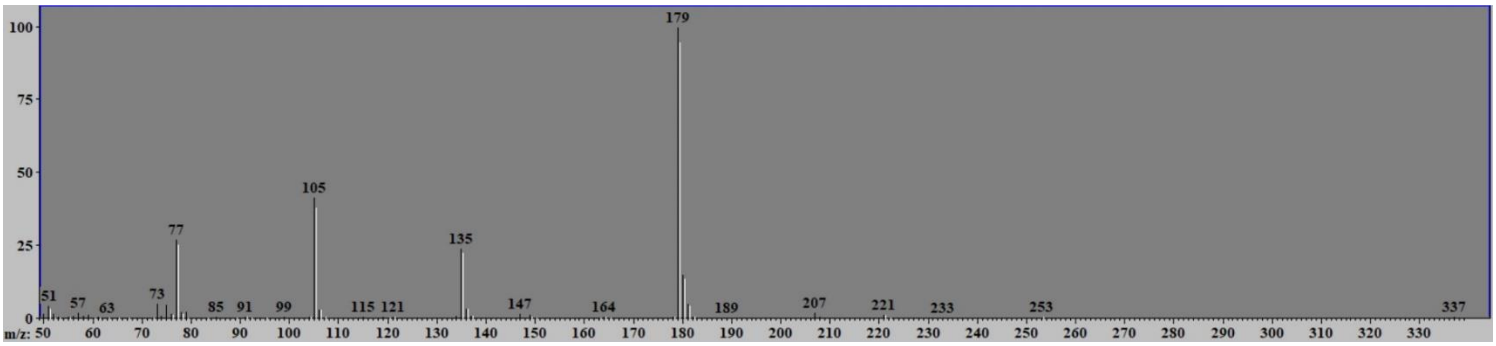

Figure A.10. MS of the tBDMS derivative Benzoic acid.

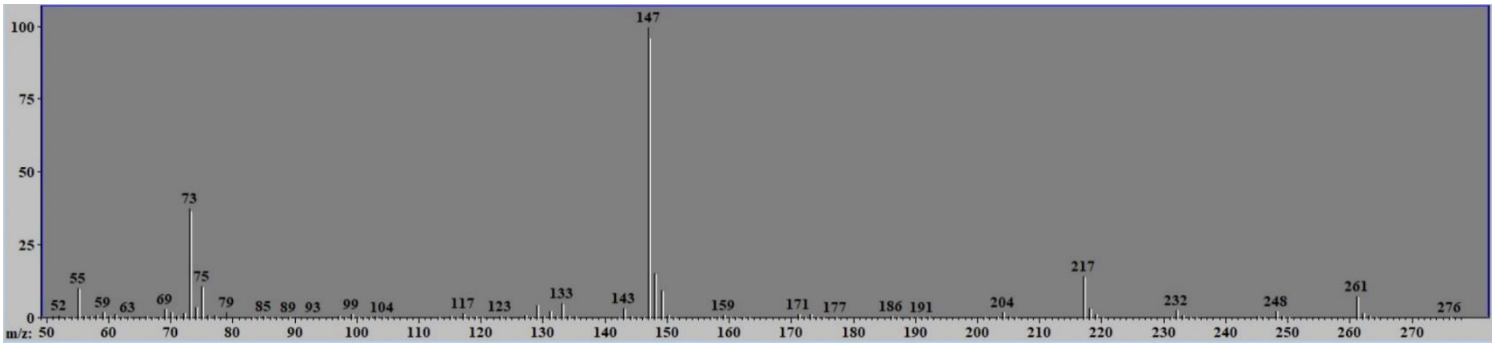

Figure A.11. MS of the TMS derivative Ethylmalonic acid.

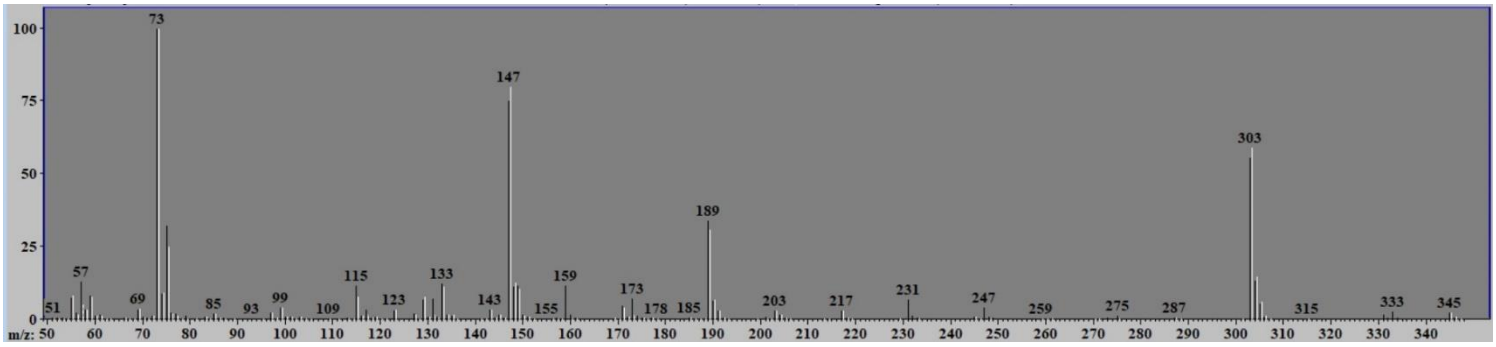

Figure A.12. MS of the tBDMS derivative Ethylmalonic acid.

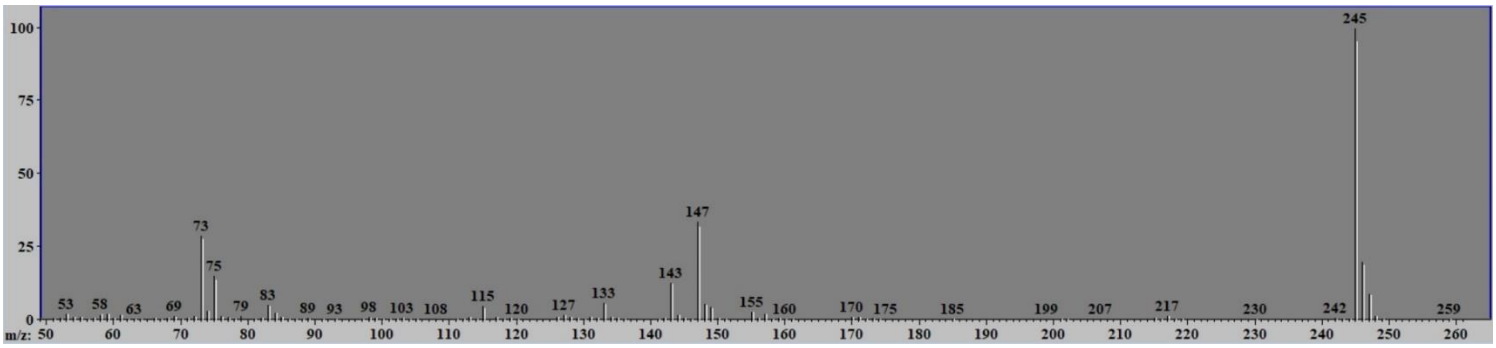

Figure A.13. MS of the TMS derivative Fumaric acid.

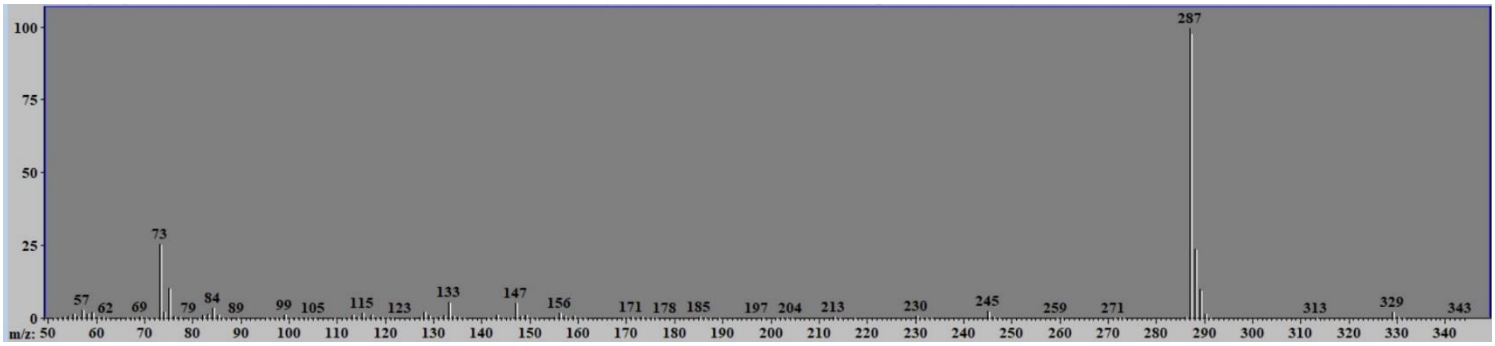

Figure A.14. MS of the tBDMS derivative Fumaric acid.

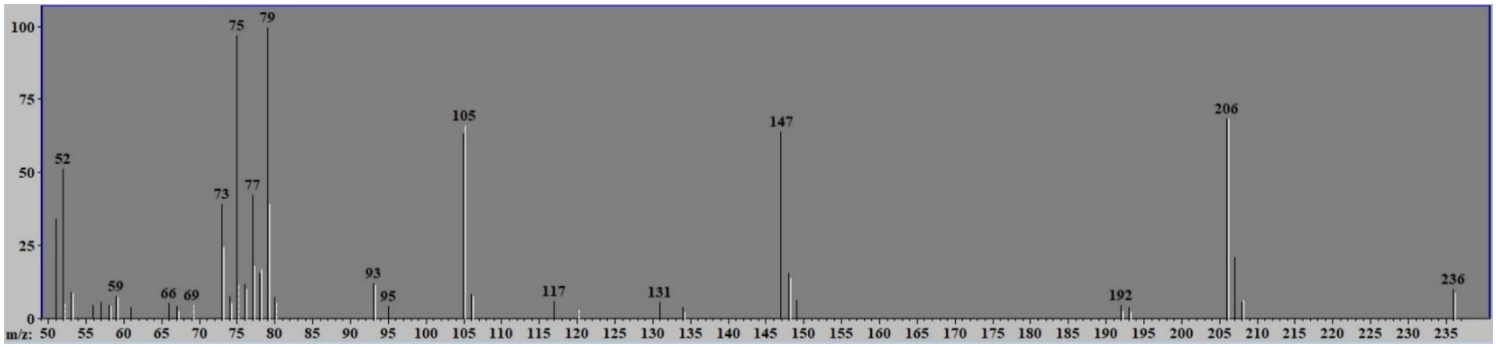

Figure A.15. MS of the TMS derivative Hippuric acid.

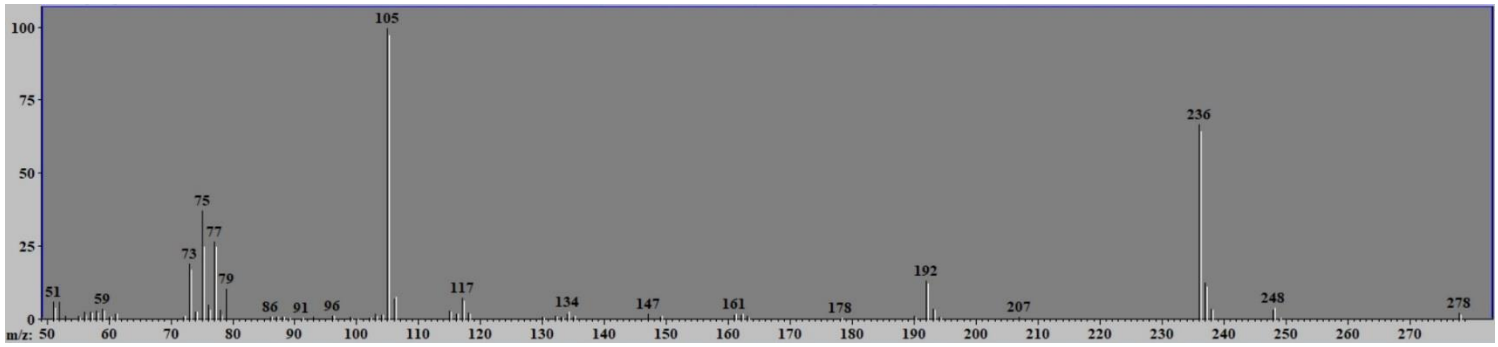

Figure A.16. MS of the tBDMS derivative Hippuric acid.

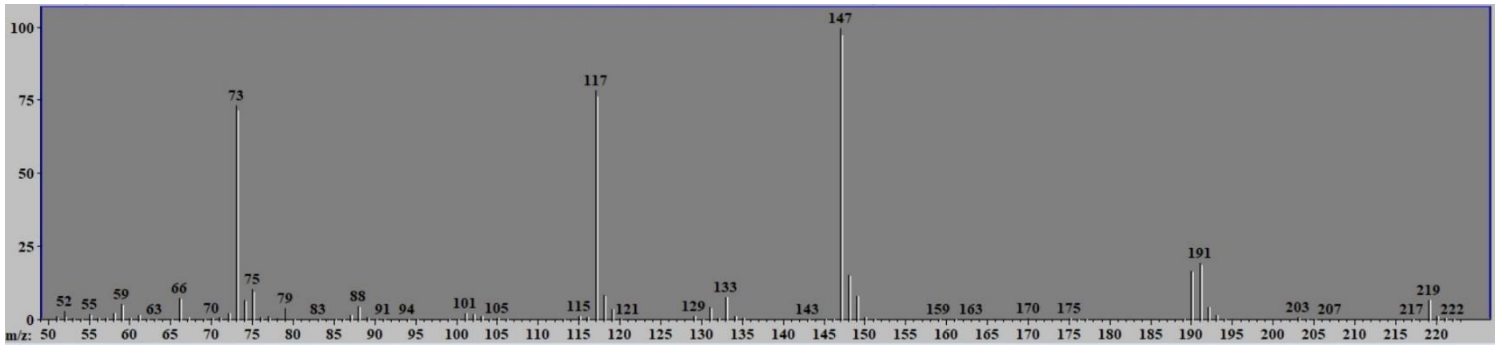

Figure A.17. MS of the TMS derivative Lactic acid.

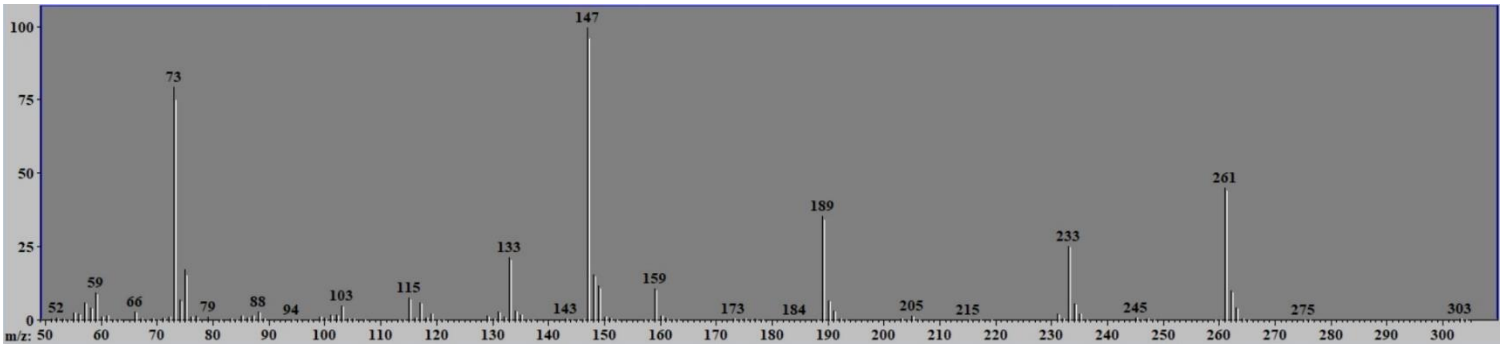

Figure A.18. MS of the tBDMS derivative Lactic acid.

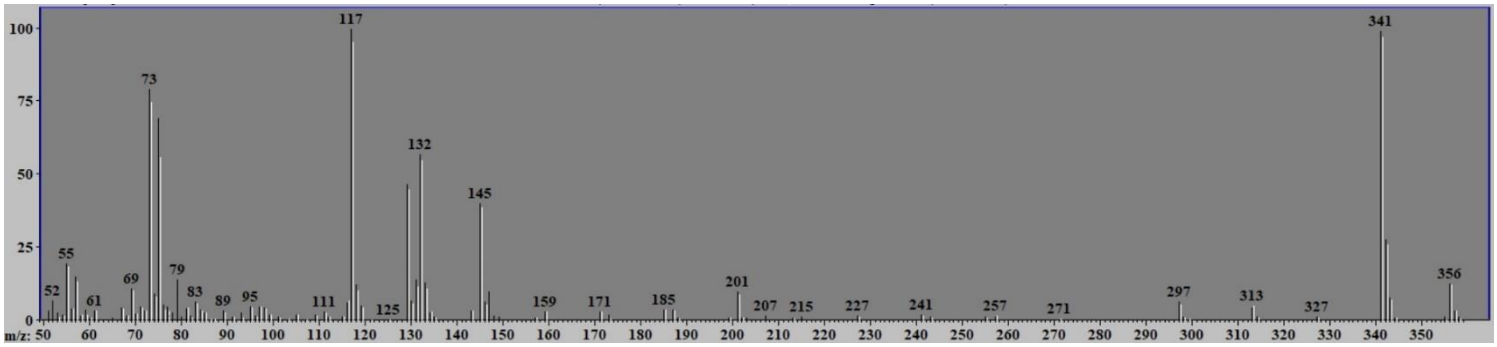

Figure A.19. MS of the TMS derivative Stearic acid.

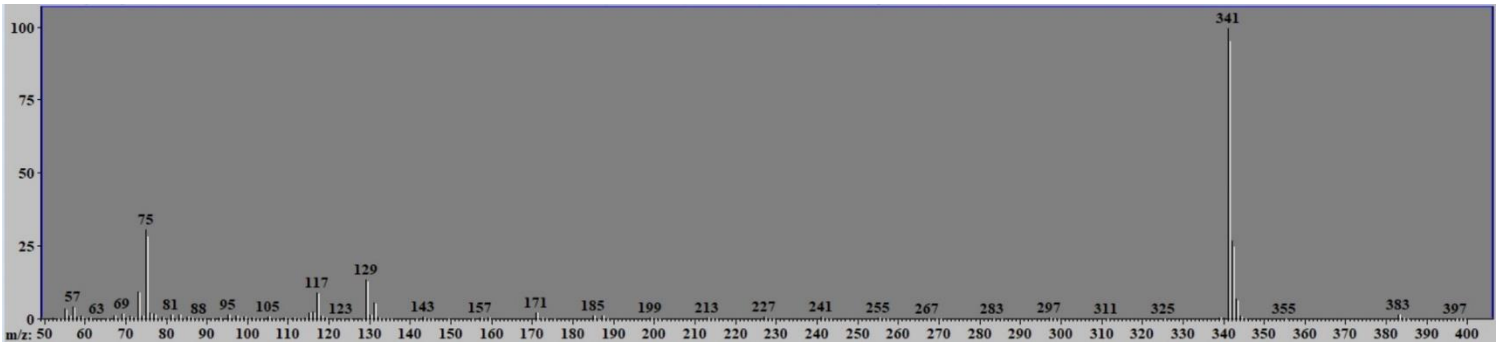

Figure A.20. MS of the tBDMS derivative Stearic acid.

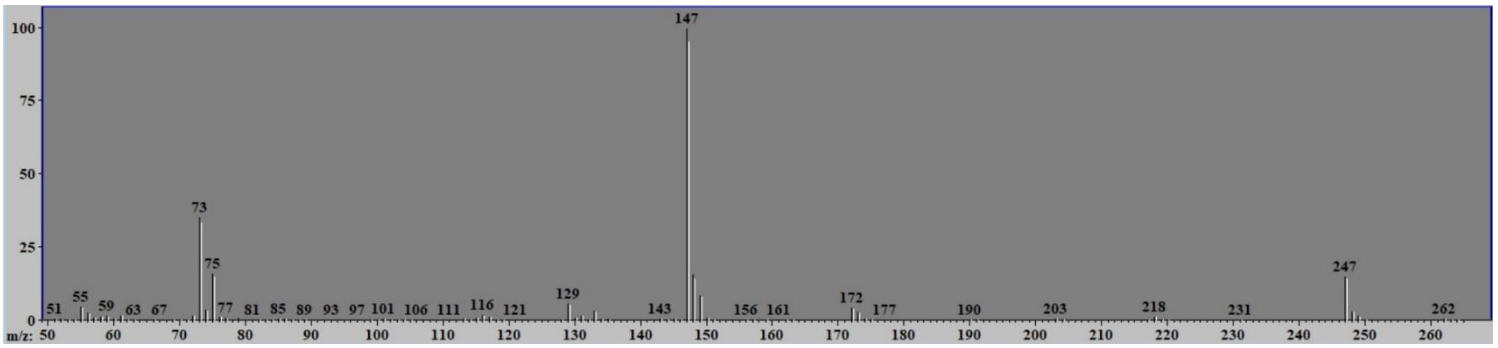

Figure A.21. MS of the TMS derivative Succinic acid.

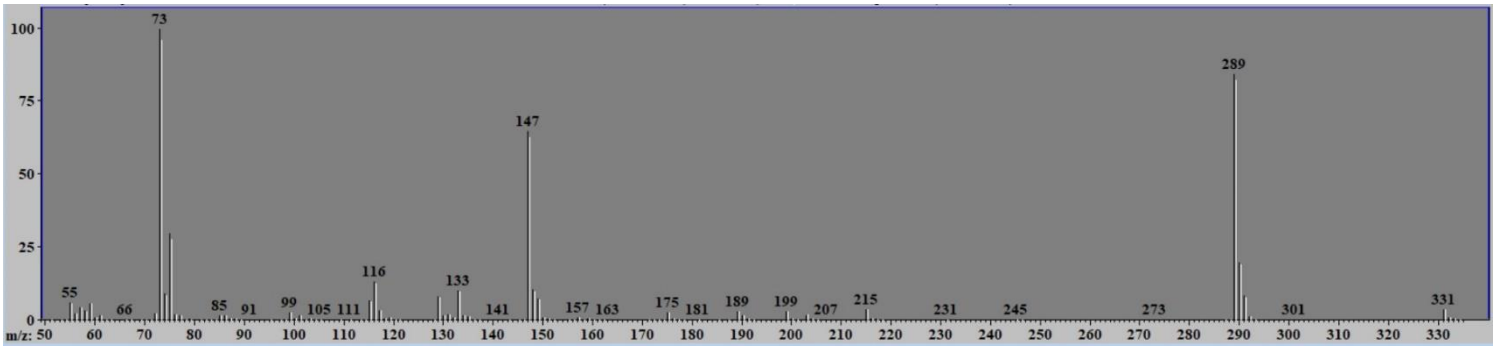

Figure A.22. MS of the tBDMS derivative Succinic acid.

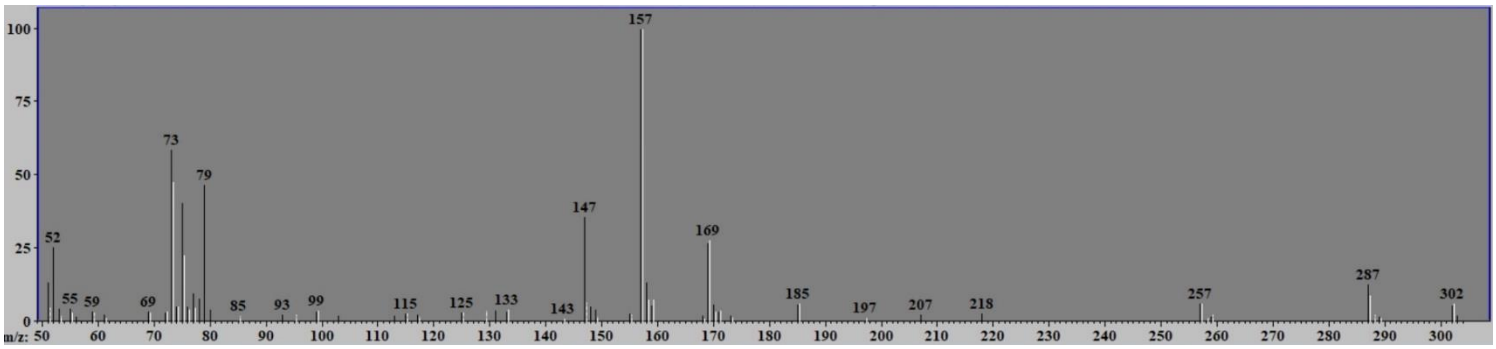

Figure A.23. MS of the TMS derivative Succinylacetone.

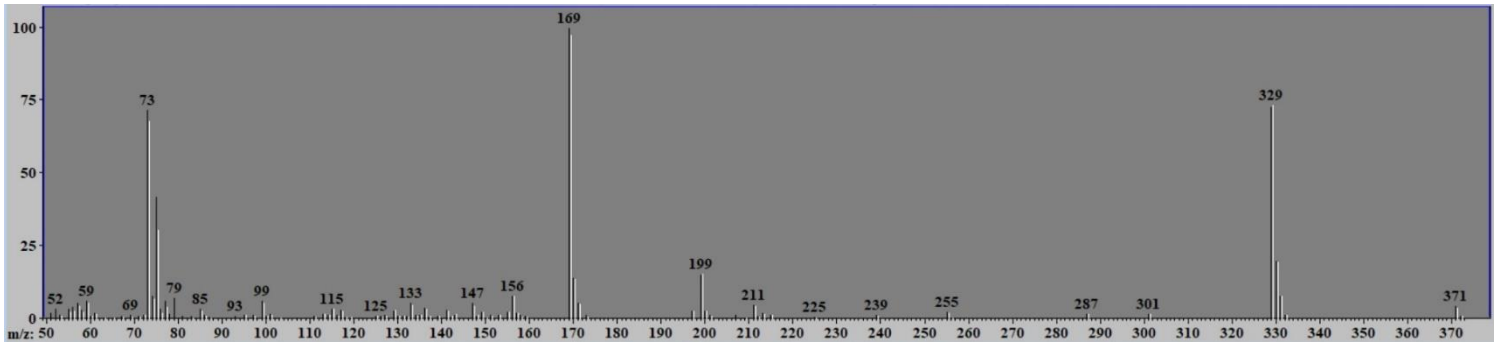

Figure A.24. MS of the tBDMS derivative Succinylacetone.

B.1 Evaluation of the number of peaks and mass spectrum

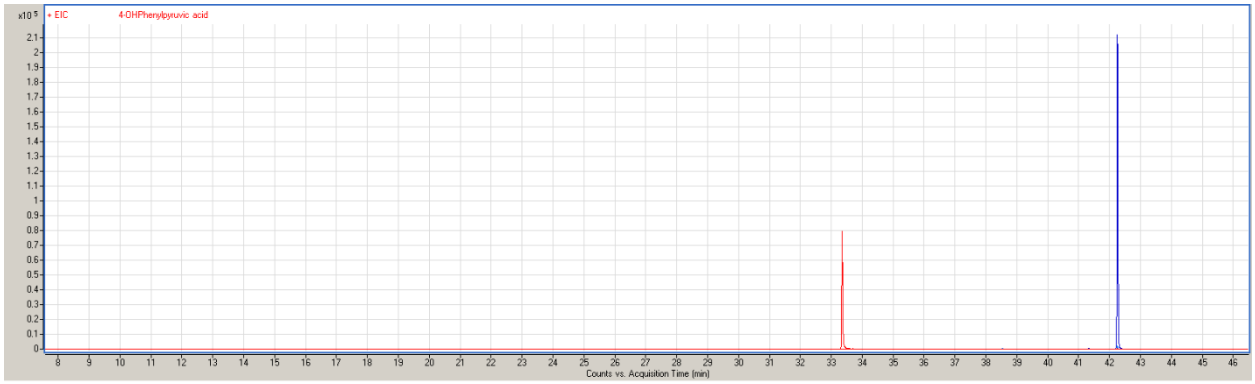

Figure B.1: Chromatogram of the fragment ions of TMS (red line) and tBDMS (blue line) derivatives of 4-Hydroxyphenylpyruvic acid. *The peak abundance of the tBDMS derivative is distinctly higher than the TMS derivative.*

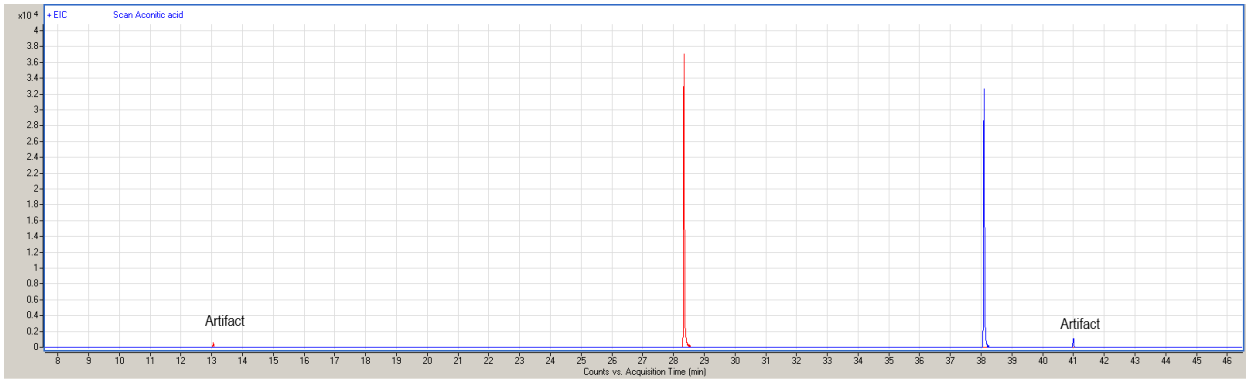

Figure B.2: Chromatogram of the fragment ions of TMS (red line) and tBDMS (blue line) derivatives of Aconitic acid. *The peak abundance of the derivative TMS is slightly higher than the tBDMS derivative.*

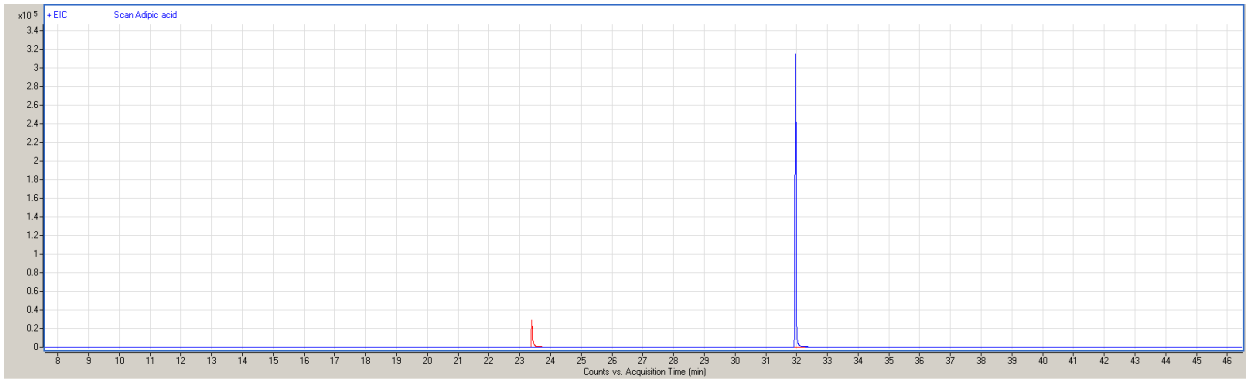

Figure B.3: EIC of the fragment ions of TMS (red line) and tBDMS (blue line) derivatives of

Adipic acid. *The peak abundance of the tBDMS derivative is distinctly higher than the TMS derivative.*

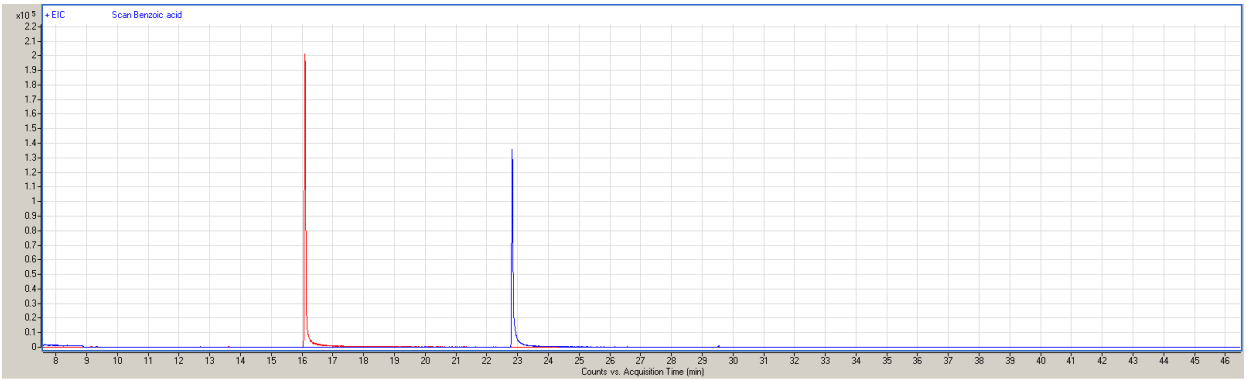

Figure B.4: EIC of the fragment ions of TMS (red line) and tBDMS (blue line) derivatives of Benzoic acid. *The peak abundance of the derivative TMS is slightly higher than the tBDMS derivative.*

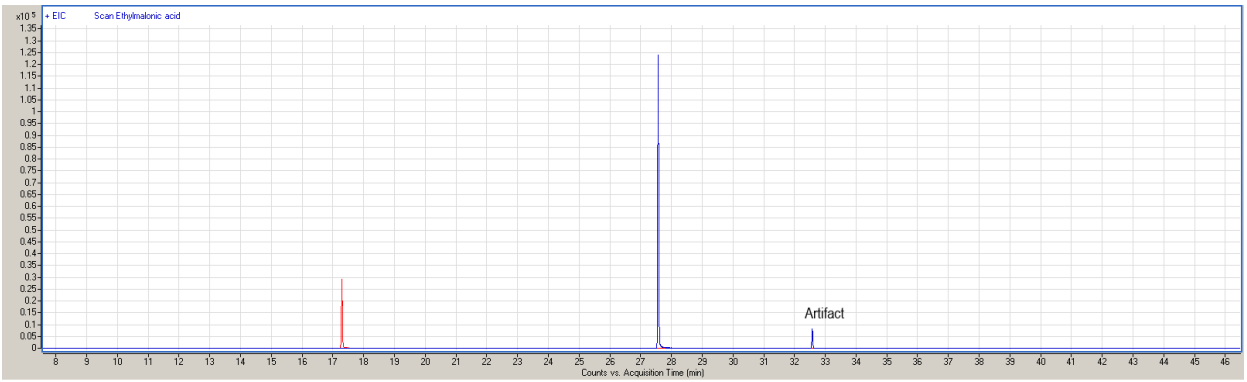

Figure B.5: Chromatogram of the fragment ions of TMS (red line) and tBDMS (blue line) derivatives of Ethylmalonic acid. *The peak abundance of the tBDMS derivative is distinctly higher than the TMS derivative.*

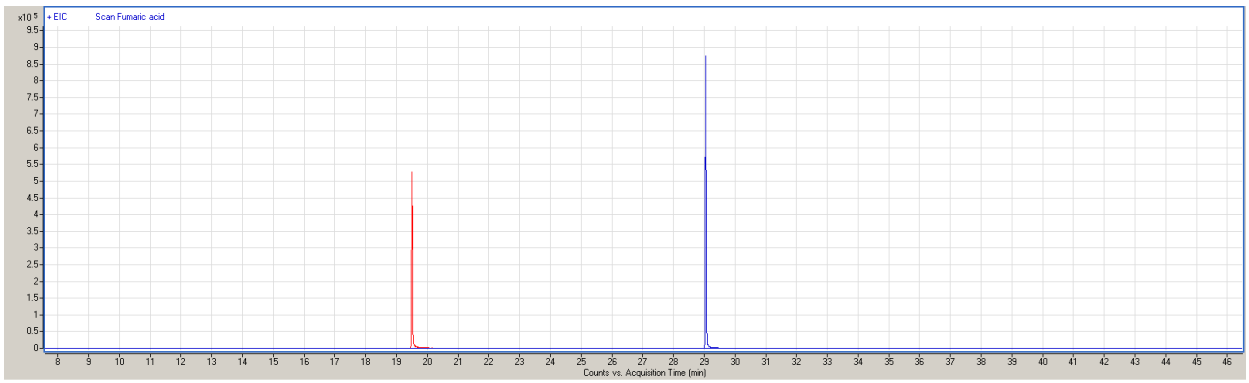

Figure B.6: EIC of the fragment ions of TMS (red line) and tBDMS (blue line) derivatives of Fumaric acid. *The peak abundance of the tBDMS derivative is distinctly higher than the TMS derivative.*

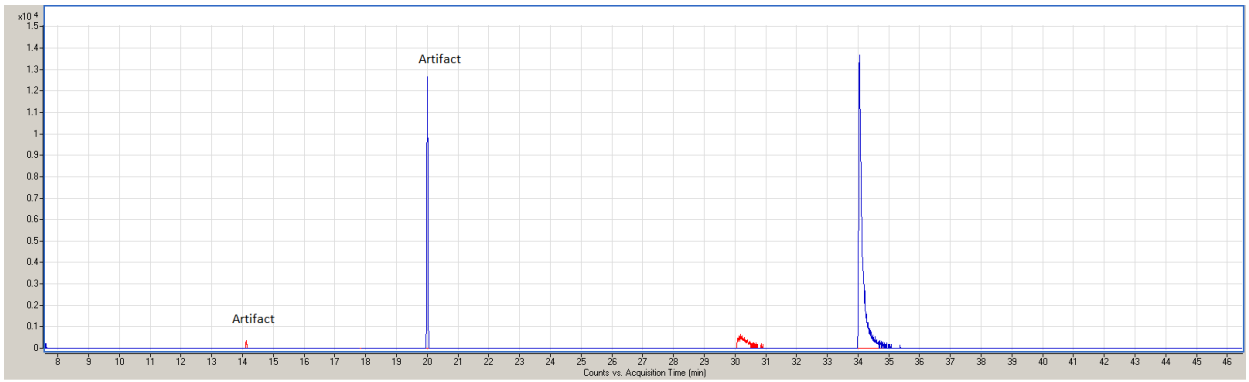

Figure B.7: EIC of the fragment ions of TMS (red line) and tBDMS (blue line) derivatives of Hippuric acid. *The peak abundance of the tBDMS derivative is distinctly higher than the TMS derivative.*

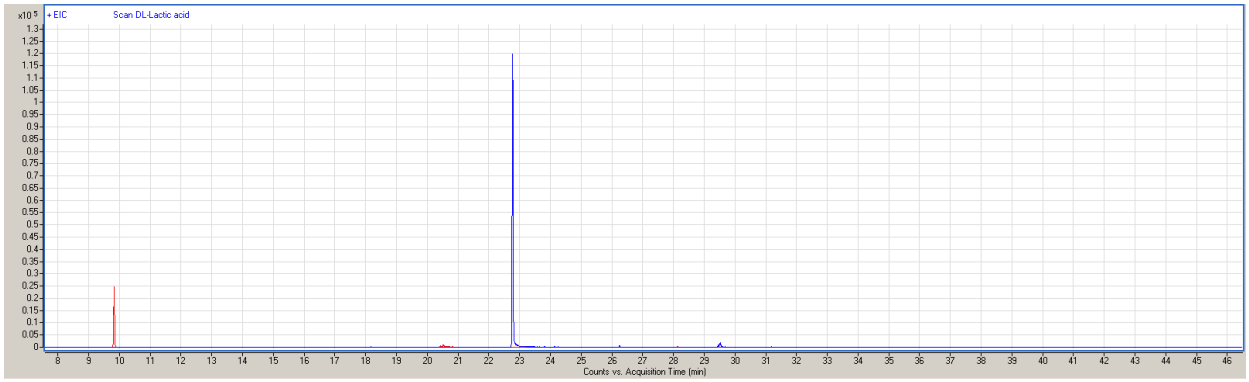

Figure B.8: EIC of the fragment ions of TMS (red line) and tBDMS (blue line) derivatives of Lactic acid. *The peak abundance of the tBDMS derivative is distinctly higher than the TMS derivative.*

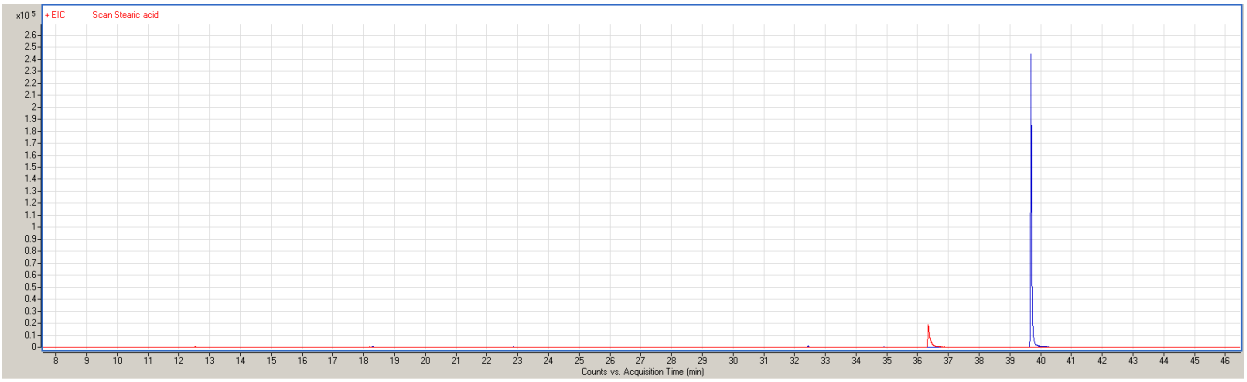

Figure B.9: EIC of the fragment ions of TMS (red line) and tBDMS (blue line) derivatives of Stearic acid. *The peak abundance of the tBDMS derivative is distinctly higher than the TMS derivative.*

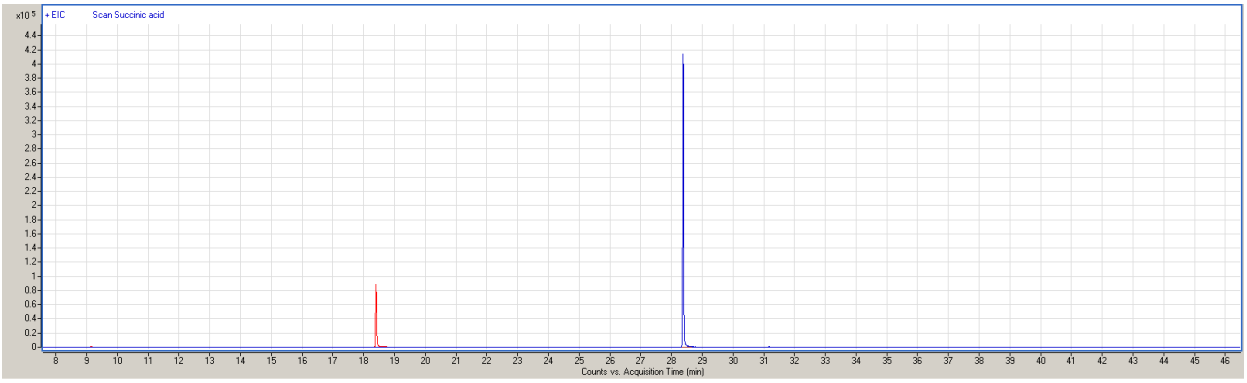

Figure B.10: EIC of the fragment ions of TMS (red line) and tBDMS (blue line) derivatives of Succinic acid. *The peak abundance of the tBDMS derivative is distinctly higher than the TMS derivative.*

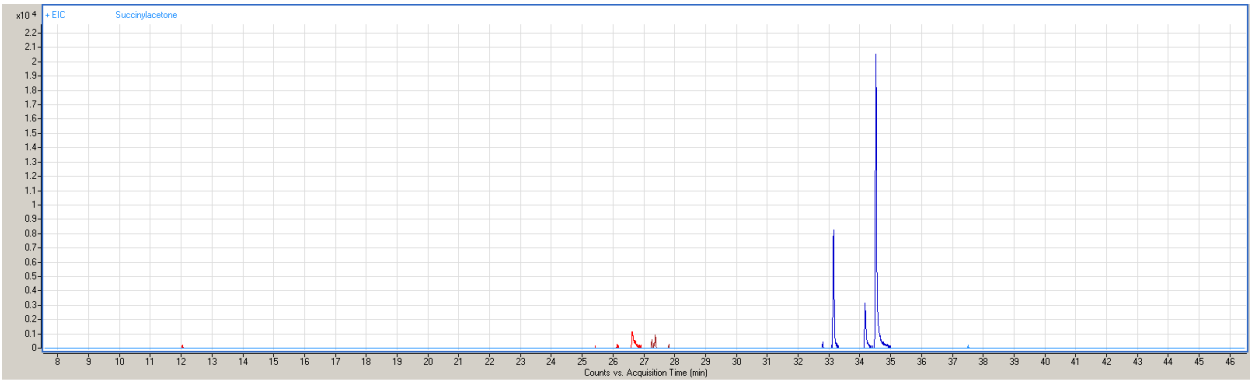

Figure B.11: EIC of the fragment ions of TMS (red and maroon line) and tBDMS (blue lines) derivatives of Succinylacetone. *The peak abundance of the tBDMS derivative is distinctly higher than the TMS derivative. Red lines = 2TMS. Maroon lines = 3TMS, Dark blue lines = 2tBDMS and light blue line = 3tBDMS.*

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