**University of South Bohemia in České Budějovice**

**Faculty of Science**


# Mass spectrometric analysis of tricarboxylic acid cycle metabolites

Bachelor Thesis

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**Annotation:**

Tricarboxylic acid (TCA) cycle is a complex metabolic hub maintained and coordinated with other metabolic pathways. Comprehensive determination of key metabolites involved in the TCA cycle is therefore of great importance in biological and biochemical research. In this study, two sample preparation approaches were examined. Silylation and in situ derivatization/extraction with ethyl chloroformate/ethanol/pyridine aqueous medium were tested. The ethyl chloroformate-based approach has been found as highly perspective.

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## Acknowledgement

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## Abstract

Tricarboxylic acid (TCA) cycle is a complex metabolic hub maintained and coordinated with other metabolic pathways. Altered metabolism in the TCA cycle has serious consequences for the physiological and metabolic state of each organism and its development. Comprehensive determination of key metabolites involved in the TCA cycle is therefore of great importance in biological and biochemical research. In this study, two sample preparation approaches, silylation and in situ derivatization- extraction with ethyl chloroformate/ethanol/pyridine aqueous medium were examined for simultaneous gas chromatographic – mass spectrometric analysis of eight relevant metabolites of the TCA cycle and pyruvic acid. While silylation method was found laborious and tedious, the latter approach enabled simultaneous GC-MS analysis of eight metabolites and pyruvate, except unstable oxaloacetate. The arising products provide defined chromatographic peaks and finger-print electron impact mass spectra enabling the simultaneous metabolite analysis and their unequivocal identification in less than 15 min. In summary, the ethyl chloroformate-based approach has been found a method of choice and highly perspective in future investigations of the TCA cycle in complex biological matrices.

## Table of Contents

## List of Abbreviations

| | |
|---|---|
| CE | capillary electrophoresis |
| DCM | dichloromethane |
| DMF | dimethylformamide |
| DMSO | dimethyl sulfoxide |
| ECF | ethyl chloformate |
| EI | electron impact |
| ESI | electrospray ionization |
| EtOH | ethanol |
| GC | gas chromatography |
| LC | liquid chromatography |
| MHA | methylhydroxylamine hydrochloride |
| MS | mass spectrometry |
| MTBSTFA | N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide |
| MW | molecular weight |
| RCF | alkyl chloroformate |
| RT | retention time |
| TBDMS | tert-Butyldimethylsilyl |
| TBDMSCl | tert-Butyldimethylchlorosilane |
| TBDMSIM | 1-(tert-Butyldimethylsilyl)imidazole |
| TCA cycle | tricarboxylic acid cycle |
| TMS | trimethylsilyl |
| TMSI | trimethylsilylimidazol |

# 1. Introduction

## 1.1. Role of tricarboxylic acid cycle in metabolism

Tricarboxylic acid (TCA) cycle, also known as citric acid cycle or Krebs cycle, is a central metabolic hub of the cell, important in energy production and biosynthesis, Fig. 1. It is an aerobic process and lack of oxygen causes its total or partial inhibition[1]. In prokaryotes the TCA cycle is located in cytosol, while in eukaryotes the cycle takes place in semi-fluid mitochondrial matrix. The operation of the cycle is enhanced by association of metabolically related enzymes into metabolons that facilitate channelling of substrates through selected sets of enzymes[2]. The semi-fluid character of mitochondrial matrix, folded membranes, proteins, and RNA and DNA molecules also facilitates kinetic and spatial compartmentation[3].

The oxidation of acetyl-coenzyme A (Ac-CoA) to $CO_2$ is central process in energy metabolism. It is yielded from catabolism of glucose, fatty acids, and some amino acids. The dominant source of acetyl-coenzyme A is oxidative decarboxylation of pyruvate catalyzed by pyruvate dehydrogenase complex (PDH complex) at the end of glycolysis. The acetyl group of the acetyl-coenzyme A enters the cycle via condensation reaction with oxaloacetate. In the course of eight oxidation-reduction reactions, the acetyl group is oxidized to two molecules of carbon dioxide. The carbons in the $CO_2$ molecules are not identical to those entering the cycle in the form of acetyl group[4]. Energy released at each turn of the cycle is stored in three NADH, one $FADH_2$ along with one molecule of adenosine triphosphate (ATP). The electrons arising from the reduced electron carriers are transferred to oxygen and the energy of the electron flow is fixed in ATP molecules during oxidative phosphorylation. The reactions, metabolites and products of the TCA cycle are shown in Fig. 1.

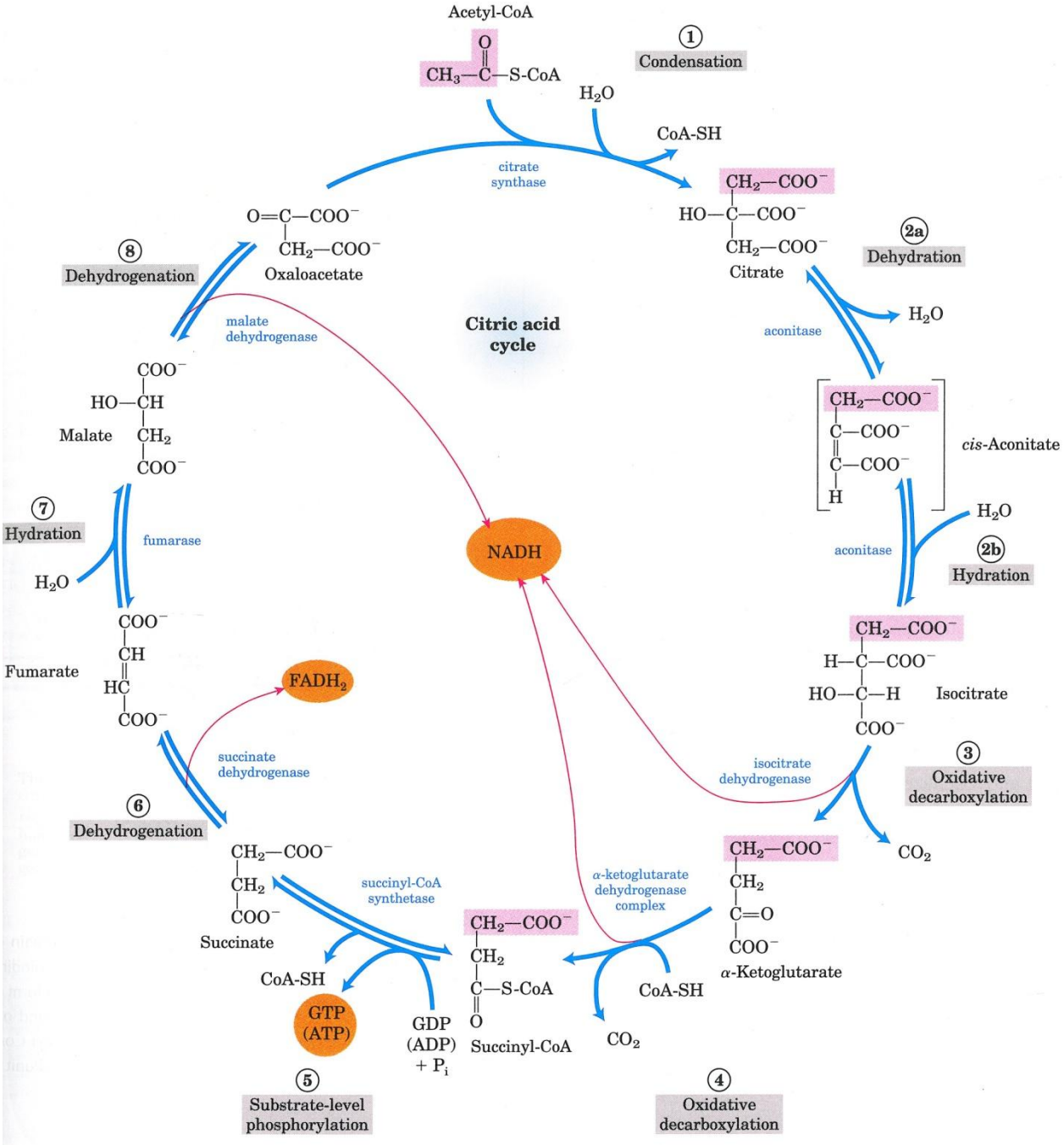



**Fig. 1:** Reactions of the TCA cycle. The pink shaded carbon atoms are derived from the acetyl group of acetyl-coenzyme A. It is impossible to distinguish the carbons in/after succinate, because symmetry of the molecule. Adapted from [4].

The TCA cycle is an amphibolic metabolic pathway: it participates both in catabolic and anabolic processes. Due to this ambiguity, the cycle serves as source of energy, but also provides variety of important biosynthetic precursors. For example oxaloacetate is a starting material for gluconeogenesis and together with α-ketoglutarate also serves as molecular building block for many amino acids, as well as for purine and pyrimidine nucleotides. Fatty acid biosynthesis starts via citrate. Succinyl-coenzyme plays an important role in the biosynthesis of porphyrin rings in heme[4].

The TCA cycle must be maintained and coordinated with other metabolic pathways that enter and leave this major turntable of the cell metabolism[3, 5]. The processes replenishing intermediates into the cycle are called anaplerotic reactions (anaplerosis). The opposite process by which an intermediate in the TCA cycle is removed to prevent its accumulation in the mitochondrial matrix is called cataplerosis[6].

Reversible carboxylation of pyruvate with $CO_2$ to oxaloacetate is a notable example of anaplerosis. The reaction is catalysed by pyruvate carboxylase and mostly takes place in liver and kidney of mammals. During starvation, amino acids also serve as source of energy and some of them they enter the cycle via the analoplerotic reactions. Under normal conditions, balance between anaplerosis and cataplerosis facilitates to hold concentrations of intermediates in the cycle at almost constant level and maintains optimal activity of the cycle[4]. The regulation of anaplerosis and cataplerosis depends upon the metabolic and physiological stage of the individual and its specific organs/tissues involved. The importance of the anaplerotic and catapletoric reactions further underlines their role that both pathways play in the regulation of glucose, nonessential amino acid and fatty acid metabolism[6, 7].

Observations of the altered metabolism in the TCA cycle and association of anaplerotic and cataplerotic metabolic pathways has been subjected to intense research, because of key role of these processes in organism and disease development. Consequently, sensitive and efficient analytical methods are highly desirable for simultaneous analysis of acidic metabolites involved in the TCA cycle.

### 1.2. How to analyse the intermediates of the TCA cycle

The intermediates are present at various concentrations in a complex biological matrix, inside the cell, in the cell tissues or body fluids. The matrix represents a complex, buffered aqueous biological environment with a number of prospective components involving inorganic ions, macromolecules such as lipoproteins, proteins, glycans and organels and membrane structures that may interfere in the metabolite analysis.

Various analytical methods have been investigated for the simultaneous analysis of the metabolites involved in the TCA cycle at the same time, using an automated electrochemical analyser[8], enzymatic assay kit available for citrate, isocitrate, succinate, malate, oxaloacetate and 2-ketoglutarate[9], by liquid chromatography with ultraviolet (UV) detection[10] or fluorometric detection[11], by ion-exchange chromatography coupled to mass spectrometry (LC-MS) [12] by capillary electrophoresis coupled to mass spectrometry (CE-MS)[13,14] and by gas chromatography coupled to mass spectrometry (GC-MS) upon prior derivatization of analytes with silylation reagents or alkyl chloroformates. For the simultaneous assay of the acidic metabolites the GC-MS technique has been most frequently applied. Its sample preparation always requires a derivatization step involving blockage of protic functional groups[15]. In this study, GC-MS methodology was preferred for investigations of simultaneous analysis of the TCA cycle intermediates. For the metabolites investigated in the study see Appendix 1.

### 1.3. Preparation of biological samples with chemical derivatization

Sample preparation is an essential step prior to analysis of small molecule metabolites in a complex biological matrix. The preparation usually involves various extraction procedures with respect to the chemical structure and thus polarity of the targeted analytes. Liquid-liquid extraction (LLE) and solid phase extraction (SPE) represent most common strategies for these purposes. However, acidic metabolites of the TCA cycle are small molecules possessing mostly carboxylic, hydroxyl- and keto- groups that are difficult to analyse simultaneously. With this respect, an efficient separation system and mass spectrometer as a detector have been most appropriate, because enable both unequivocal identification as well robust and cost-effective quantitation of metabolites. In addition to LC-MS[11-12] and CE-MS methods[13-14], GC-MS has been more accessible technique requiring only a routine instrumentation for analytical

measurements of acidic analytes. However, chemical derivatization of acids is an essential step in this case and relies mainly on silylation[16-18] or derivatization with alkyl chloroformates[19-21].

Derivatization was introduced into analytical chemistry to obtain desired product of the analyte with optimal properties via chemical reaction proceeding between the original sample and a derivatization reagent. Specific derivatization reactions can be performed before the chromatographic separation (pre-column), during (on-column) and between the separation and introduction of analyte into a detector (post-column)[22].

In the field of GC-MS analysis, chemical derivatization enables to increase volatility, temperature stability a improve separation properties of the analytes. Low volatility is caused by high molecular weight of the analyte, but more often by the presence of polar protic groups (-COOH, OH, -NH, and -SH) in the structure resulting in the strong intermolecular interactions that deteriorate the GC separation process [23, 24].

### 1.4. Silylation

Acidic metabolites have mostly been treated with the silylation reagents, the most common are summarized in Table 1.

Silylation is one of the most versatile derivatization techniques used in gas chromatography. Silyl derivatives are formed according to scheme shown in Fig. 2.

The reaction with trimethylsilyl group was used as example. The –COOH, -OH, -SH, and –NH groups are the protic target undergoing the derivatization reaction. The arising derivatives exhibit enhanced volatility and thermal stability. Their polarity is decreased.

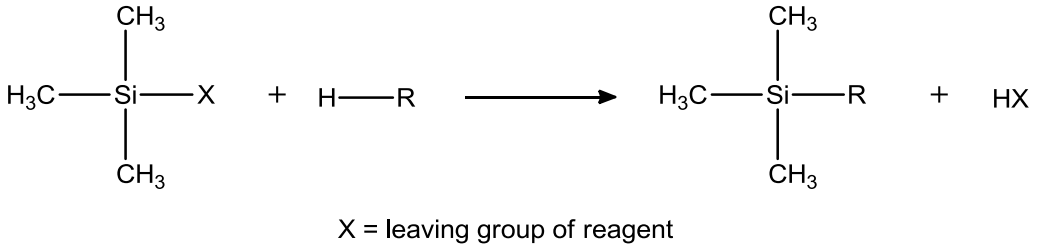


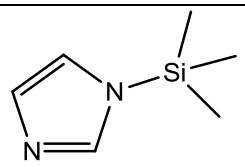
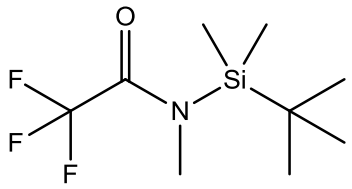
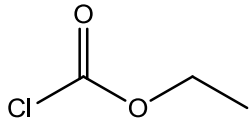
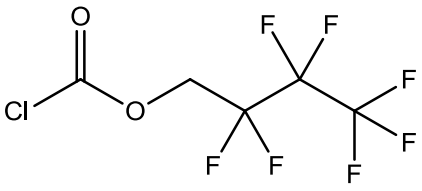

**Fig. 2:** A scheme of the trimethylsilyl derivatization reaction

Nowadays, silylation reagents – donors of the trimethylsilyl (TMS) group and a bulkier tert-butyldimethylsilyl (TBDMS) group have been used. The derivatives containing bulkier

groups are generally more stable, but the lower reactivity is a disadvantage of the reagents possessing the TBDMS group. Abilities of different functional groups to react with a silylation reagent follow the order: alcoholic hydroxyl > phenolic hydroxyl > carboxyl > amine > amide[23]. The reactivity of a particular reagent toward each compound class is also influenced by the steric hindrance of their functional groups. The primary alcohol is the most reactive one, whereas the tertiary alcohol is the least reactive. The order is similar in case of amines: primary group is silylated much easier than the secondary amine. Unprotected keto groups form hydrolytically and thermally unstable keto-enol derivatives. In many instances the keto groups are therefore protected by formation of an oxime or O-alkyloxime[25].

The silylated derivatives are sensitive to hydrolysis and thus residues of water have carefully to be removed from a sample before the reaction with a silylation reagent. If it is not possible, excess of the reagent can be added and moisture is removed from the sample by hydrolysis of of water residues with the silylation reagent. Reagents containing sterically hindered groups such as TBDMS can alternatively be employed. Silylation reactions are typically carried out in aprotic solvents such as pyridine, dimethylformamide (DMF), dimethyl sulphoxide (DMSO), tetrahydrofuran (THF) and acetonitrile. In some cases silylation reagent itself can be used as suitable solvent[25]. The reaction time is in (minutes - hours) and temperature (25 °C - 100 °C or higher) of silylation reaction varies depending on the analyte structure and the reactivity of the reagent.

**Table 1:** List of common derivatization reagents used in GC-MS of acidic metabolites

| Silylation reagents | |
|---|---|
| 1-(Trimethylsilyl)imidazole (TMSI) |  |
| N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA)<br><br>N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide + 1% tert-Butyldimethylchlorosilane, (MTBSTFA + 1% TBDMSCl) |  |
| Derivatization with chloroformates | |
| Ethyl chloroformate (ECF) |  |
| Heptafluorobutyl chloroformate (HFBCF) |  |

### 1.5. Derivatization with alkyl chloroformates

In contrast to silylation, derivatization with alkyl chloroformates (RCF) proceeds directly in an aqueous medium, in situ[19-21], typically in the presence of the corresponding alcohol and under pyridine catalysis.

Carboxyl, hydroxyl, thiol and amino groups thus can simultaneously be protected according to schemes shown in Fig. 2, where reactions with phenol (yielding the carbonate) and glycine (providing the corresponding ester and carbamate) are used as examples.

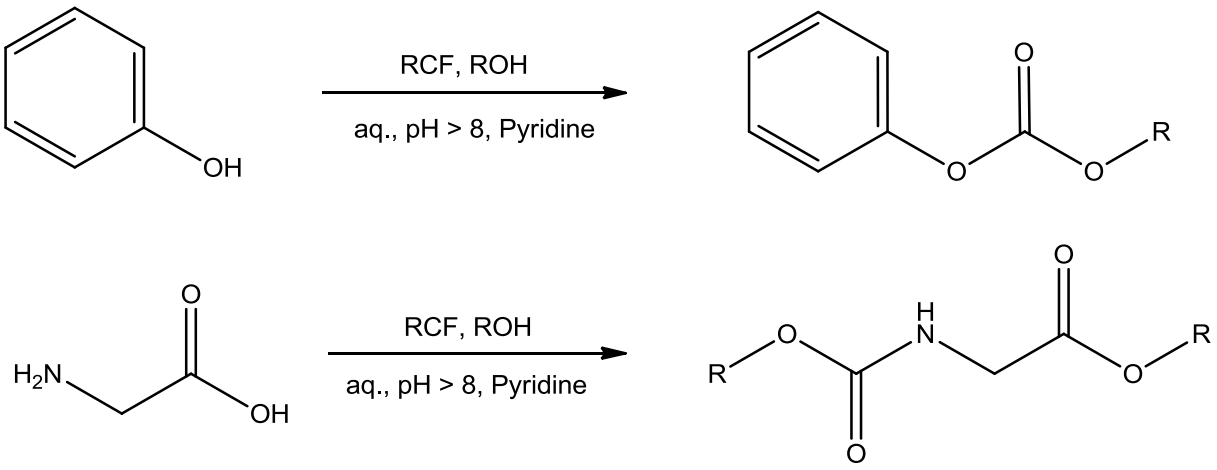



**Fig. 3:** Scheme of phenol and glycine derivatization with an alkyl chloroformate (RCF)

The arising derivatives are much less polar and easily extractable into an immiscible organic phase. Furthermore, the derivatives obtained with alkyl chloroformates are also amenable to LC-MS analysis[26]. The derivatization reaction is very fast (5 min) and cost effective.

## 2. Aim of the thesis

- to summarized current knowledge about TCA acid cycle
- to found suitable method for analysis of metabolites involved in TCA cycle

# 3. Experimental

## 3.1. Reagents and chemicals

Ethyl chloformate (ECF), dimethylformamide (anhydrous, DMF), pyridine (anhydrous), methylhydroxylamine hydrochloride (MHA), ammonium formate, potassium hydroxide, and citric, cis-aconitic, fumaric, isocitric, α-ketoglutaric, malic, oxaloacetic, pyruvic and hydrochloric (37 %) acid were purchased from Sigma Aldrich (Praha, Czech Republic). Silylation reagents, trimethylimidazol (TMSI), 1-(tert-butyldimethylsilyl)imidazole (TBDMSIM) , N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA), and N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide + 1% tert-butyldimethylchlorosilane (MTBSTFA + 1 % TBDMSCl), were also supplied by Sigma-Aldrich (Praha, Czech Republic). Ethanol, dichloromethane (DCM), isooctane, and chloroform were purchased from Merck. Methanol was purchased from Fisher Scientific (Pardubice, Czech Republic).

## 3.2. Stock solutions

10 mM (10 μmol/mL) stock aqueous solution of each examined acid was prepared. The solution containing all examined acids was mixed from the stock solutions to the final concentration 1 mM (1 μmol/mL) of each acid.

## 3.3. Sample preparation methods

### 3.3.1. Derivatization with ethyl chloroformate (ECF)

Reactions were carried out in glass tubes 6 × 50 mm without any closures. Aliquot of a sample stock solution was used and filled up with water to 100 μL. Ethanol - pyridine mixture (75 μL, 4:1, v/v) and the same volume of chloroform – ECF (9:1, v/v) were vortexed with the sample solution to create emulsion. Then, 75 μL of 1.5 M NaOH were added for pH adjustment and the content was vortexed. An aliquot of chloroform – ECF (75 μL, 9:1, v/v) was again added, followed by vortexing and centrifugation. The final mixture was treated with 75 μL of 3 M HCl, properly mixed and centrifuged.

For GC-MS analysis, 50 μl of the sample extract was taken from the bottom organic phase, transferred into a 1.1 ml autosampler vial and 0,5 μl aliquot was injected into an GC injection port of the GS-MS spectrometer. Another 30 μL aliquot of the bottom organic layer was

transferred into another vial and carefully evaporated to dryness under a mild stream of $N_2$ at room temperature. The residue was immediately dissolved in 60 µL of a mobile phase and analysed by LC-MS.

### 3.3.2 Derivatization with oximation - silylation reagents

The derivatization procedure was performed in thick-wall glass vials with open-top screw caps and PTFE/Rubber laminated discs (Supelco, Sigma-Aldrich). An aqueous solution of each sample was evaporated to dryness with a 100 µL dichloromethane by a mild stream of nitrogen. The portion of DCM was added in excess to facilitate evaporation of moisture from the sample. The oximation reaction step was performed with 25 µL MHA solution (fresh, 20 mg MHA in 1 mL pyridine) in 30 µL of dried dimethylformamide. The content was mixed and heated at 80°C in an oven for 30 minutes.

The subsequent silylation step was accomplished with addition of 70 µL of dried dimethyl-formamide and 30 µL of silylation reagent were added to the sample (cooled to room temperature). The content was vortexed for 5 s. Then the mixture was incubated at 80°C for 30 minutes in an oven and then cooled to room temperature. Finally, 150 µL of isooctane was added, followed by proper vortexing. After separation of two immiscible layers, the upper organic layer extract was withdrawn, transferred in an autosampler vial and analysed by GS-MS.

## 3.4. Instrumental analysis

### 3.4.1. GC-MS analysis

GC-MS analysis was performed using programmable temperature vaporizing injector connected to a ThermoFisher Scientific Trace GC Ultra and Trace DSQ single quadrupole mass spectrometer equipped with EI ion source (all Thermo Scientific, USA). Xcalibur 2.1 software (Thermo Scientific, USA) was used for GC-MS system control, data acquisition and data processing.

### 3.4.2. LC-MS analysis

LC-MS analysis was carried out on a linear ion trap mass spectrometer LTQ XL (Thermo Scientifics, USA) equipped with an electrospray ion source and connected to an HTC PAL

system autosampler (CTC Analytics, Switzerland) and Rheos Allergo pump (Flux Instruments, Switzerland). Xcalibur 2.1 software (Thermo Scientific, USA) was used for LC-MS system control, data acquisition and data processing.

### 3.4.3. GC-MS analysis of the ECF derivatized products

A 0.5 µL aliquot was injected in the splitless mode (closed for 0.7 min, split flow: 50 mL/min) and the injector was kept at 250 °C. Properties of the used GC-columns were following: TR-50MS, 30 m × 0.25 mm i.d., 0.25 µm film thickness (Thermo Scientifics, USA) and VF-17MS, 30 m × 0.25 mm, i.d. 0.25 µm (Agilent, USA). The oven was held at 45 °C for 1.2 minute, raised at 16 °C/min until 330 °C and held for 2 min. Helium was used as carrier gas with constant flow rate 1.1 mL/min. The ion source was set to 230 °C and the MS transfer line was held at 250 °C. Detection employed EI mode in full scan regime (40-500 Da).

### 3.4.4. LC-MS analysis of the ECF derivatized products

The injection volume was set to 5 µL and a column was kept at 35 °C. LC column Kinetex C18, 150 mm × 2.1 mm, 2.6 µm (Phenomenex) was used to achieved a separation with mobile phase consisting of methanol (A) and water (B), both enriched with 5 mM ammonium formate. The gradient elution was as follows: A/B = 0 min : 30/70, 12 min : 100/0. The flow rate was set to 200 µL/min. The following parameters were used for ESI: capillary temperature of 200 °C and vaporizer temperature of 150 °C, source voltage of 4 kV for positive and of 3 kV for negative ionization modes, capillary voltage of 40 V for positive ionization mode and – 40 V for negative ionization mode. Nitrogen was used as the desolvation-declustering gas.

### 3.4.5. GC-MS analysis of the oximation-silylation products

A 1 µL of the sample extract aliquot was injected in splitless mode (closed for 0.7 min, split flow: 20 mL/min) and the injector was kept at 220 °C. Properties of the used GC-column were following: VF-17MS, 30 m × 0.25 mm, i.d. 0.25 µm (Agilent, USA). The oven was held at 40 °C for 1 min, than raised at 5 °C/min until 60 °C and subsequently ramped to 302 °C with rate 12 °C/min and held for 2 min. Helium was used as carrier gas with constant flow rate 1.1 mL/min. The ion source was set to 200 °C and the MS transfer line was held at 250 °C. Detection employed EI mode in full scan regime (50-750 Da).

# 4. Results and Discussion

## 4.1. TCA cycle metabolites derivatized with ECF/EtOH/pyridine/chloroform medium

### 4.1.1. GC-MS investigations

The metabolites were treated with ECF. The derivatized products were extracted into an organic layer and then separated on a fused capillary column. A typical extracted chromatogram of a standard mixture obtained by GC-MS analysis is presented in Fig. 4. The retention time and three most abundant m/z signals of each derivative are summarized in Table 2.

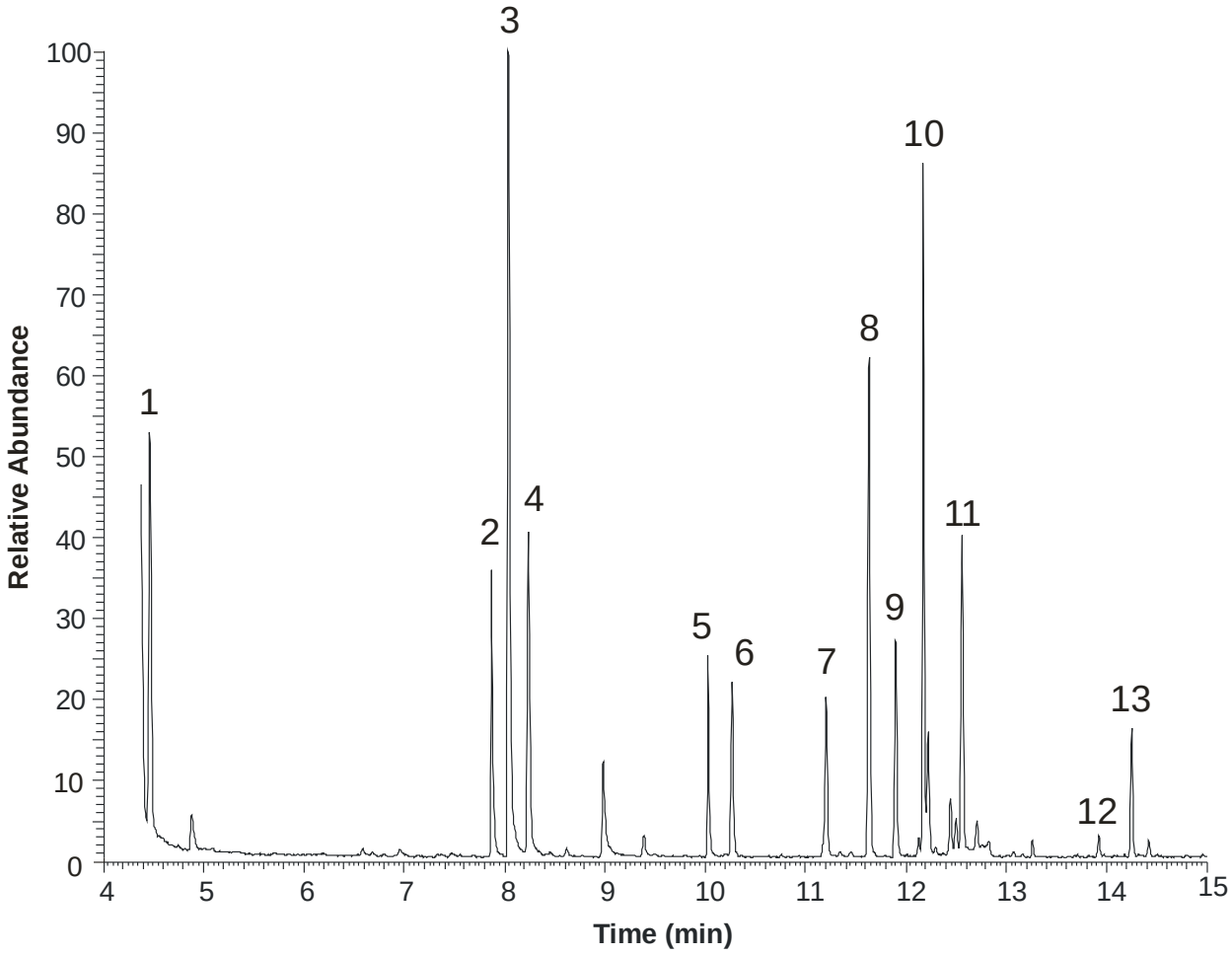



**Fig. 4:** GC-MS chromatogram showing separation of analytes on a TR-50MS column (ThermoScientific, 30 m × 0.25 mm, 0.25 µm film thickness) after treatment with ECF. 1 = pyruvic acid, 2 = fumaric acid, 3 = succinic acid, 4 = maleic acid, 5 = α-ketoglutaric acid (1), 6 = α-ketoglutaric acid (2), 7 = citric acid (1), 8 = malic acid, 9 = cis-aconitic acid, 10 = citric acid (2), 11 = isocitric acid (1), 12 = citric acid (3), 13 = isocitric acid (2)

**Table 2:** GC-MS analysis; retention times and three most abundant m/z signals of detected analytes after derivatization with ECF.

| Analyte | RT | diagnostic m/z ions (% of rel. abundance)[a] | | |
|---|---|---|---|---|
| | | m/z (100) | m/z | m/z |
| **Pyruvic acid** | 4.46 | 43 | 42 (6) | 45 (6) |
| **Fumaric acid** | 7.87 | 127 | 99 (63) | 126 (37) |
| **Succinic acid** | 8.04 | 101 | 129 (62) | 55 (24) |
| **Maleic acid** | 8.24 | 99 | 127 (27) | 126 (17) |
| **α-Ketoglutaric acid (1)** | 10.03 | 101 | 129 (51) | 55 (26) |
| **α-Ketoglutaric acid (2)** | 10.27 | 101 | 129 (66) | 55 (27) |
| **Citric acid (1)** | 11.20 | 157 | 115 (93) | 43 (57) |
| **Malic acid** | 11.64 | 71 | 117 (65) | 89 (48) |
| **cis-Aconitic acid** | 11.90 | 112 | 212 (62) | 213 (55) |
| **Citric acid (2)** | 12.17 | 157 | 115 (35) | 203 (17) |
| **Isocitric acid (1)** | 12.56 | 129 | 157 (90) | 101 (83) |
| **Citric acid (3)** | 13.93 | 157 | 115 (57) | 213 (49) |
| **Isocitric acid (2)** | 14.25 | 157 | 129 (87) | 101 (44) |

[a] Diagnostic m/z ions suitable for identification involve the most intensive m/z (100 %) and other 2 fragment ions.

For the EI spectrum of each particular detected metabolite derivative see Appendix 2.

Pyruvic acid and all organic acids participated in the TCA cycle were clearly detected, except oxaloacetic acid. The oxaloacetic acid derivative was not detected in the GC-MS chromatograms although various reaction, extraction, and chromatographic conditions were examined with freshly prepared aqueous standard solutions. This is not surprising, because the intermediate is known to be very unstable and easily decarboxylates to pyruvate [10, 16].

Other seven acidic metabolites of the TCA cycle and pyruvic acid provide well defined derivatization products by their retention and fingerprint-type EI mass spectra. They are also searchable in commercially available mass spectral libraries such as NIST 2.0 installed on the used GC-MS spectrometer.

ECF is a highly reactive species, which efficiently esterifies carboxylic group in aqueous environment in seconds. Nevertheless, in sterically hindered structures, such as citric acid, the reaction route results in 2-4 products, which were observed on the investigated GC-MS chromatograms. Thus treatment of citric, isocitric, and α-ketoglutaric acids provided four, three and two products, respectively. Refer also to Table 2 and also their EI spectra in the Appendix 2.

In addition to the supposed tri-, di-esters forms, OH group is converted to the O-ethoxycarbonyl derivative. The further peaks in the (iso)citric acids represent the derivatives, where the sterically hindered hydroxyl group is not transformed to carbonate moiety or one of the neighbouring sterically hindered carboxyl groups remains untouched.

However, the structural elucidation has not definitively been solved yet and will be a subject of further study. Dehydratation of citric acid to cis-aconitic acid structure was also observed to a minor extent. The chromatogram of citric acid derivatives is shown in Fig. 5.

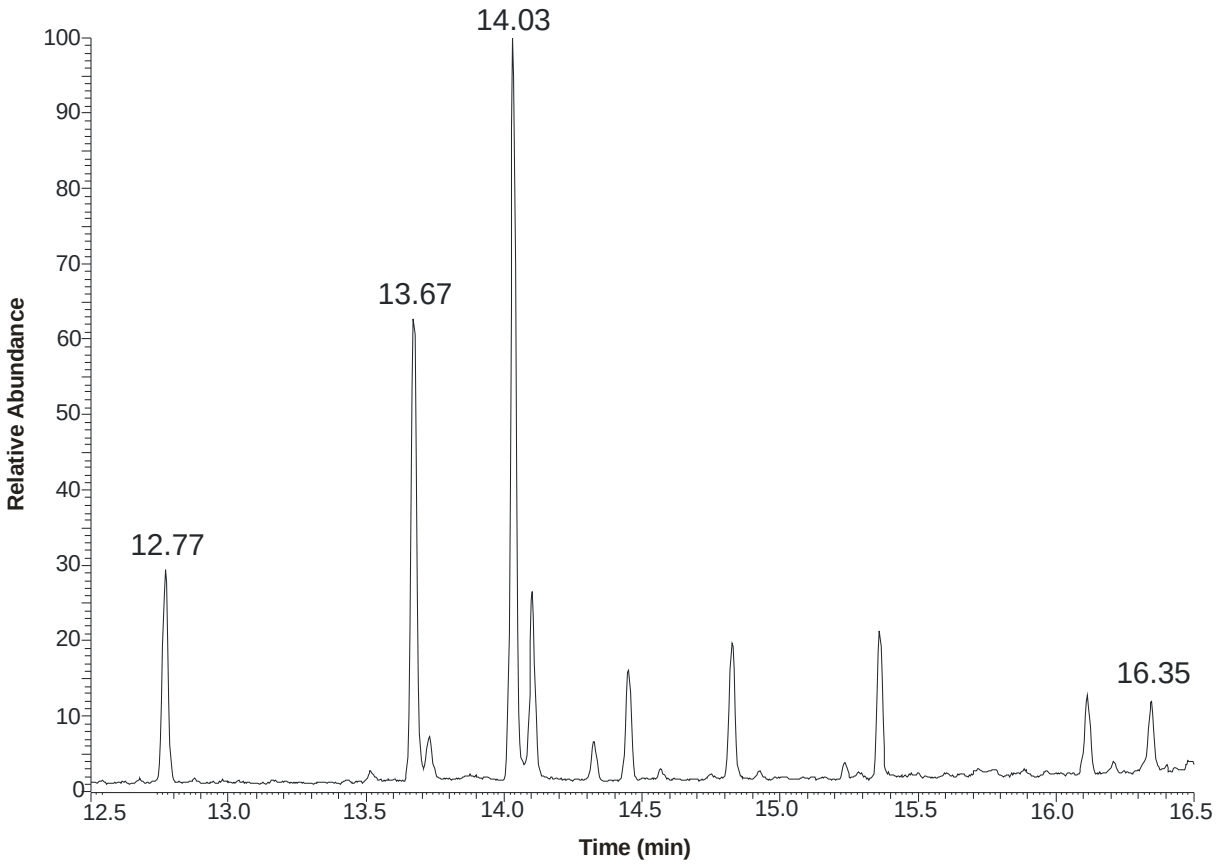



**Fig. 5:** GC-MS chromatogram of citric acid derivatives; column VF-17MS (30 m × 0.25 mm, 0.25 μm film thickness). 12.77 = citric acid (1), 13.67 = cis-aconitic acid, 14.03 = citric acid (2), and 16.35 = citric acid (3).

Interestingly, fumaric acid provided two peaks. According to their EI mass spectra, the intermediate possessing a double bond is isomerized partly into the cis-izomer - maleic acid

during the derivatization reaction. The chromatogram of fumaric acid derivatives is shown in Fig. 6.

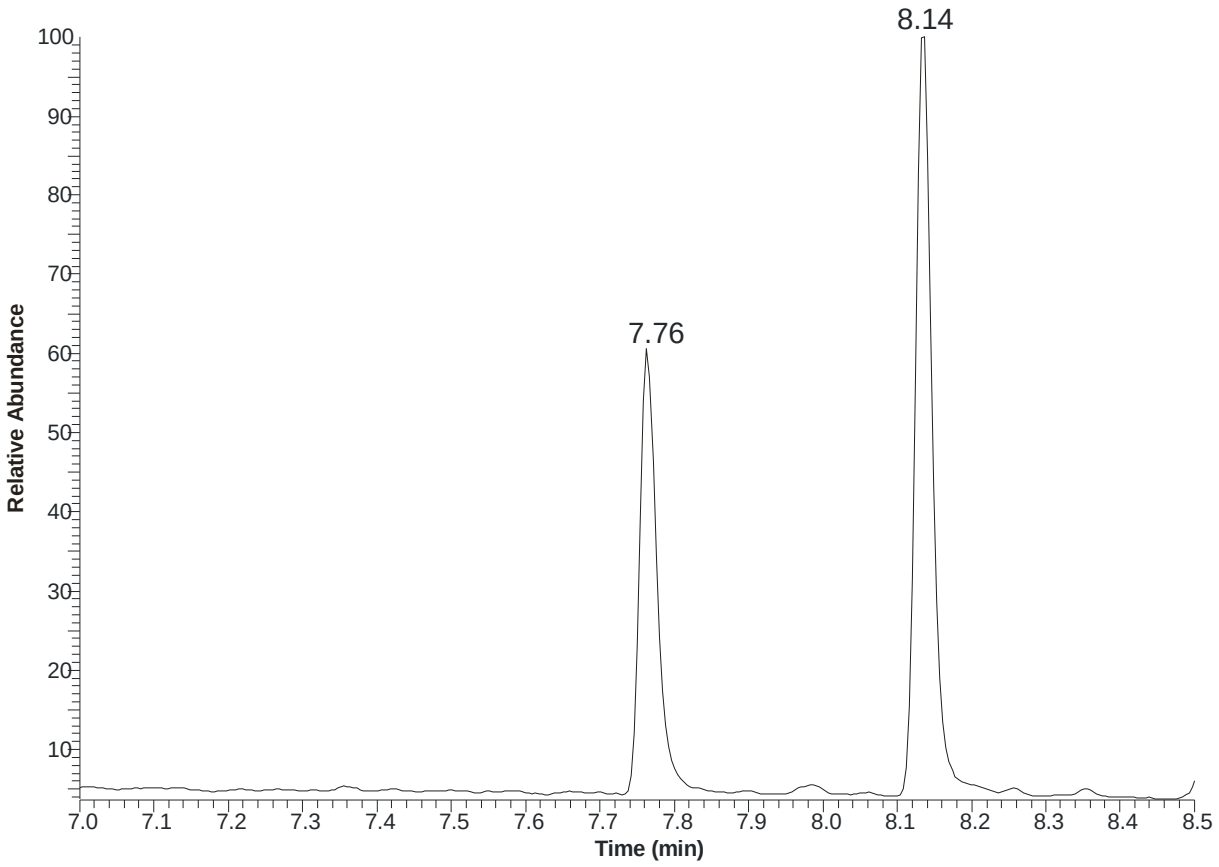



**Fig. 6:** GC-MS chromatogram of the detected fumaric acid derivatives; column TR-50MS (30 m × 0.25 mm, 0.25 µm film thickness). 7.76 = fumaric acid, 8.14 = maleic acid

### 4.1.2. LC-MS investigations

In order to confirm the structures arising from the RCF-mediated reaction, the derivatized products were also investigated by LC-MS analysis. The hydroxy- and/or oxycarbonyl ethyl esters of the studied acids are very weak bases and thus not efficient proton acceptors during electrospray ionization. As a result, only a part of them, particularly those that contain hydroxyl group or double bond, were ionized with sufficient efficiency. The detected analytes possess principal $[M + NH_4]^+$ signals on LC-MS chromatogram owing to the presence of ammonium formate buffer in the mobile phase. The LC-MS chromatogram of the detected metabolites is shown in Fig. 7.

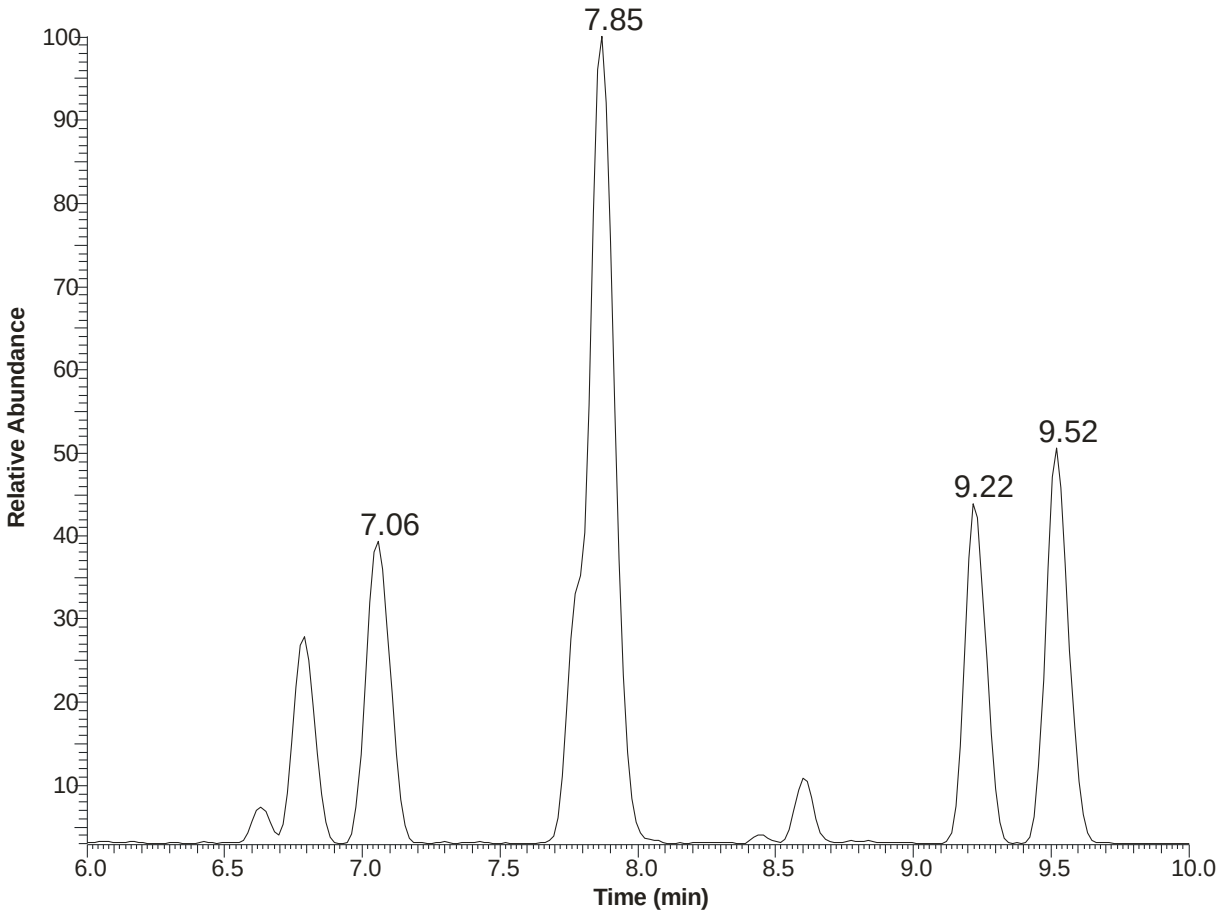



**Fig. 7:** LC-MS chromatogram showing separation of analytes on column Kinetex C18 (150 mm × 2.1 mm, 2.6 μm) after treatment with ECF. 7.06 = citric acid (1), 7.85 = malic acid, 9.22 = isocitric acid, 9.52 = cis-acinitic acid, 9.55 = citric acid (2) – coeluted with 9.52. 10 nmol of each.

Retention times, observed $[M + NH_4]^+$ ions and calculated molecular weights (MW) of the detected analytes are summarized in the Table 3.

**Table 3:** LC-MS analysis; retention times, observed $[M + NH_4]^+$ ions and the estimated molecular weight of detected analytes after derivatization with ECF.

| Analyte | RT [min] | $[M + NH_4]^+$ | MW [Da] |
|---|---|---|---|
| **Citric acid (1)** | 7.06 | 294 | 276 |
| **Malic acid** | 7.85 | 280 | 262 |
| **Isocitric acid** | 9.22 | 366 | 348 |
| **cis-Aconitic acid** | 9.52 | 276 | 258 |
| **Citric acid (2)** | 9.55 | 366 | 348 |

As documented in Table 3, four metabolites of the TCA cycle, citrate, isocitrate, malate and cis-aconitate were detected by LC-MS with positive electrospray detection. Peaks corresponding to succinate, oxaloacetate, fumarate, α-ketoglutarate and pyruvate were not observed.

For an ESI positive spectrum of each particular detected metabolite derivative, refer to the Appendix 3.

Citric acid derivatives provided two distinct peaks. Their molecular weight deduced from their ESI spectrum indicates, that all three carboxyl groups in citrate and isocitrate are successfully esterified. The detected structures of citrate differ in the treatment of hydroxyl, which remains untreated at citric acid (1) and is carbonated at citrate (2). The proposed structures of the citric acid (1) and citric acid (2) are shown in Fig. 8.

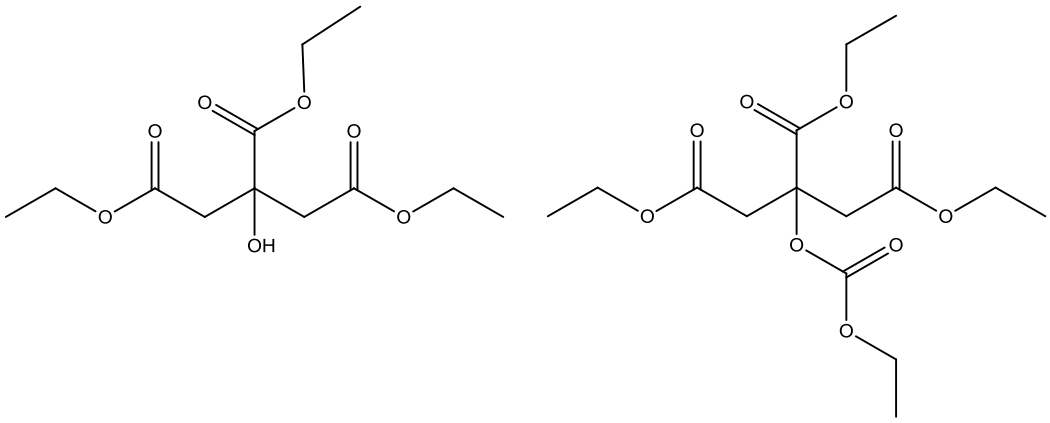



**Fig. 8:** Structures corresponding to peaks citric acid (1) and citric acid (2) in Table 2.

### 4.2. TCA metabolites derivatized with silylation reagents

The TCA metabolites contain mainly carboxy-, hydroxyl- and keto- functional groups and these groups can be directly treated with silylation reagents [25] in an non-aqueous environment. Within the study, we investigated reactions of the nine acidic metabolites with the most common oximation reagent O-methyl-hydroxylamine and four silylation reagents (TMSI, TBDMSIM, MTBSTFA, MTBSTFA + 1 % TBDMSCl). However, the obtained results were much less satisfactory than with the RCF derivatization. The sample preparation is tedious, because water has to be removed prior to the analyte treatment [23, 25]. However, even under strictly anhydrous conditions, the reaction yields were low or not obtained at all. Importantly, the reaction process cannot be coupled with liquid-liquid extraction as in the case of the ECF-

mediated derivatization method. Fouling of GC-MS instrumentation with silylation reagents and much less clean extracts is also a notable disadvantage [19-20, 23].

We tested liquid-liquid extraction of the silylated metabolites from dimethylformamide into an isooctane environment. Only derivatives of citric, fumaric and succinic acid were detected after derivatized with MTBSTFA reagent without prior oximation step. Retention times and three most abundant m/z signals of detected analytes are summarized in the Table 4.

**Table 4:** GC-MS; silylation reagent: MTBSTFA, oximation step skipped, column: VF-17 MS (30 m × 0.25 mm, 0.25 μm film thickness)

| Analyte | RT [min] | diagnostic m/z ions (% of rel. abundace)[a] | | |
|---|---|---|---|---|
| | | m/z (100) | m/z | m/z |
| **Fumaric acid** | 9.02 | 147 | 73 (74) | 289 (21) |
| **Succinic acid** | 9.11 | 287 | 73 (90) | 147 (31) |
| **Citric acid** | 14.94 | 73 | 591 (32) | 23 (17) |

[a] Diagnostic m/z ions suitable for identification involve the most intensive m/z (100 %) and other 2 fragment ions.

The failure of the silylation method and its experimented modifications was probably caused by still present moisture resulting in hydrolysis of silylation reagent and the examined derivatives of analytes. The reason of positive response of citric, fumaric and succinic acids could be found in the fact that tendency to decomposition of derivatives is different for each compound[25].

## 5. Conclusion

A series of eight metabolites involved in the TCA cycle (together with pyruvate) was investigated for simultaneous analysis by GC-MS in aqueous matrices. Two derivatization procedures were examined; in-situ derivatization with ethyl chloroformate/ethanol/pyridine aqueous medium coupled with liquid-liquid extraction of the arising derivatives into an immiscible chloroform layer and silylation with four TMS and TBDMS reagents in non-aqueous environment.

The developed ECF-mediated derivatization protocol provides well defined products of all metabolites under the study, except the unstable oxaloacetate. Although citric, isocitric, and α-ketoglutaric + fumaric acids provided four, three and two respective products, their peaks are easily separated and can be used for GC-MS simultaneously enabling thus comprehensive analysis of the intermediates of the TCA cycle.

In addition, four derivatized metabolites were also detected by positive ESI LC-MS analysis. Despite the modest ionization efficiency, the LC-MS analysis provided important information about the molecular weight of the prepared RCF derivatives and confirmed the structures of the derivatives observed by the GC-MS method.

Experiments with silylation procedures showed that this sample preparation procedure is much more laborious and requires strictly anhydrous conditions for work.

In summary, the developed ECF-mediated derivatization-extraction method has been found promising for GC-MS analysis of the acidic metabolites and will be applied to future planned investigations of the TCA cycle in cell cultures and relevant biological matrices.

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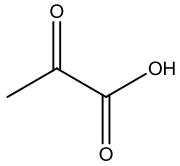
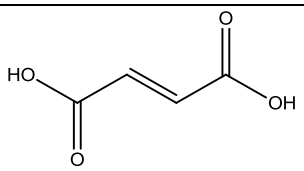
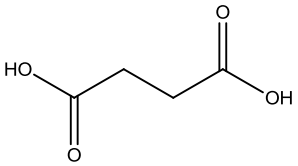
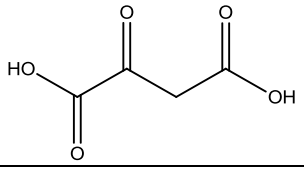
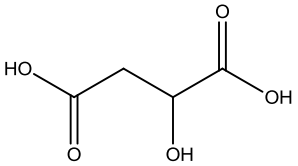
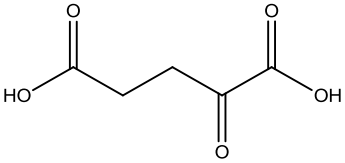
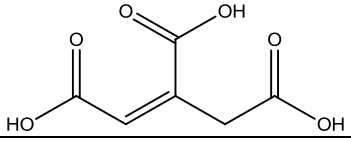
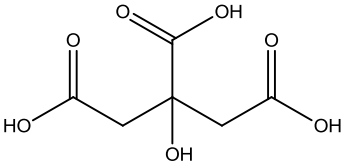
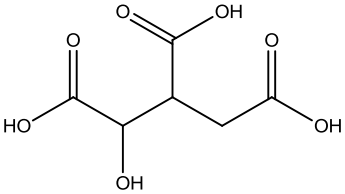
## 7. Appendix 1: Table of analytes

**Table 5:** Analytes and their molecular weights, chmical formulas and structures

| Name | MW [Da] | Chemical Formula | Chemical Structure |
|---|---|---|---|
| Pyruvic acid | 88.06 | $C_3H_4O_3$ |  |
| Fumaric acid | 116.07 | $C_4H_4O_4$ |  |
| Succinic acid | 118.09 | $C_4H_6O_4$ |  |
| Oxaloacetic acid | 132.07 | $C_4H_4O_5$ |  |
| Malic acid | 134.09 | $C_4H_6O_5$ |  |
| α-ketoglutaric acid | 146.10 | $C_5H_6O_5$ |  |
| cis-Aconitic acid | 174.11 | $C_6H_6O_6$ |  |
| Citric acid | 192.12 | $C_6H_8O_7$ |  |
| Isocitric acid | 192.12 | $C_6H_8O_7$ |  |

# 8. Appendix 2: GC-MS analysis – EI spectra of ECF derivatives

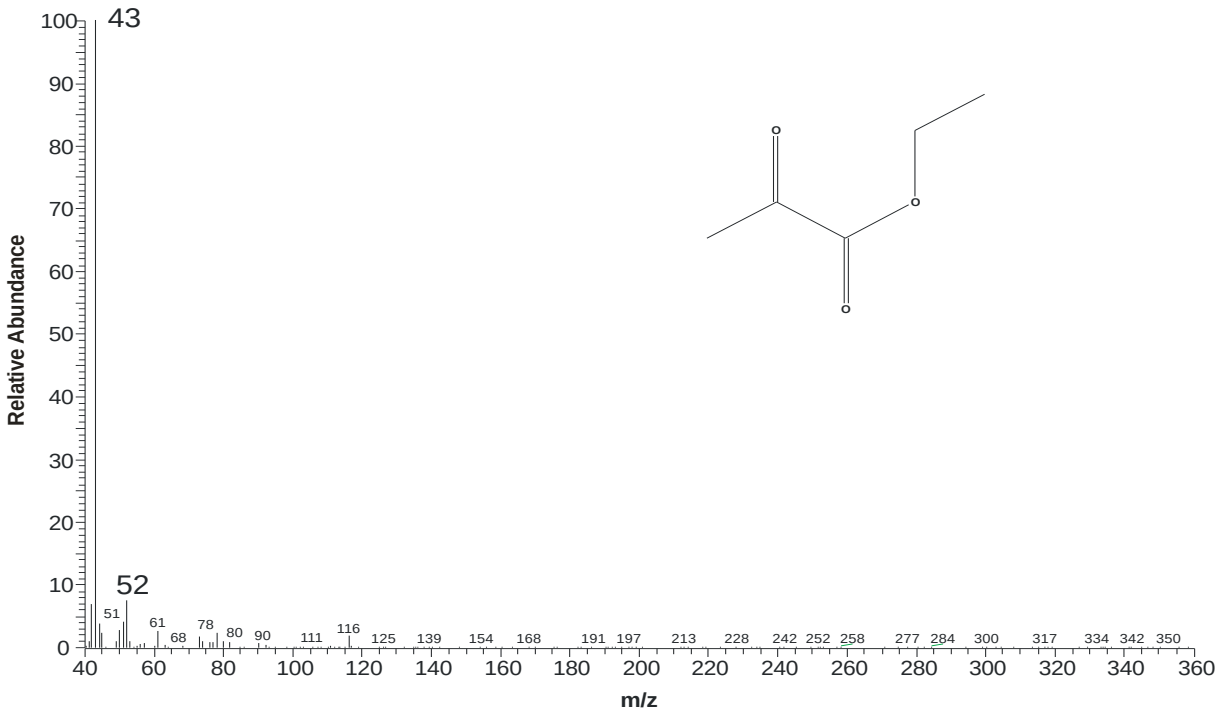



**Fig. 9:** GC-MS analysis, ECF derivatization, EI spectrum - pyruvic acid


**Fig. 10:** GC-MS analysis, ECF derivatization, EI spectrum - fumaric acid

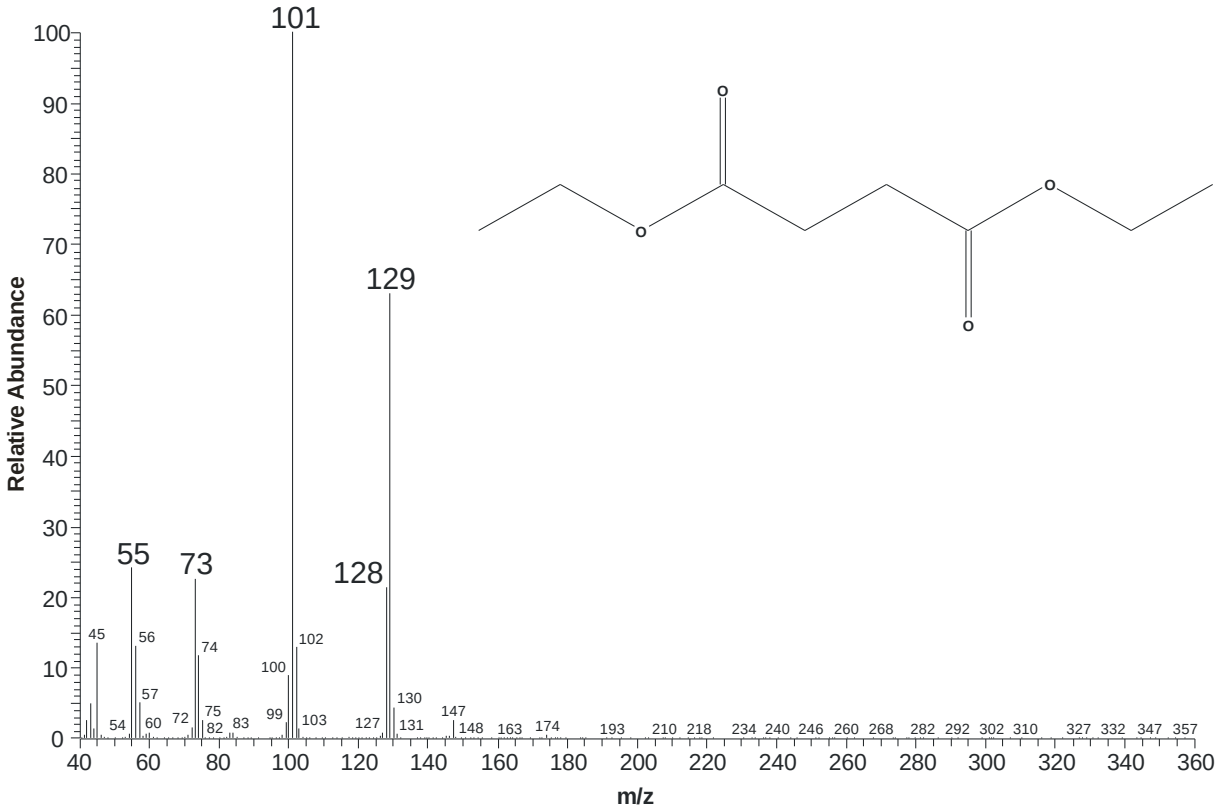



**Fig. 11:** GC-MS analysis, ECF derivatization, EI spectrum – succinic acid

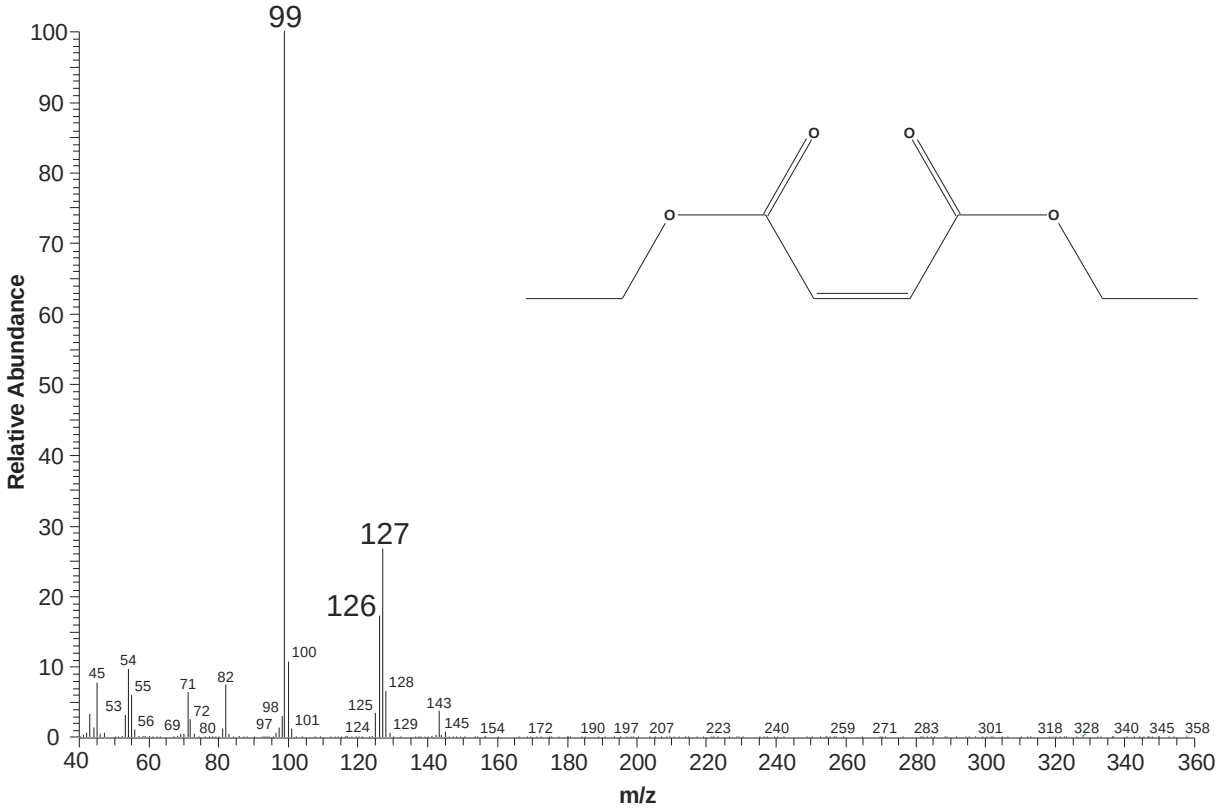



**Fig. 13:** GC-MS analysis, ECF derivatization, EI spectrum - maleic acid (from fumaric acid)

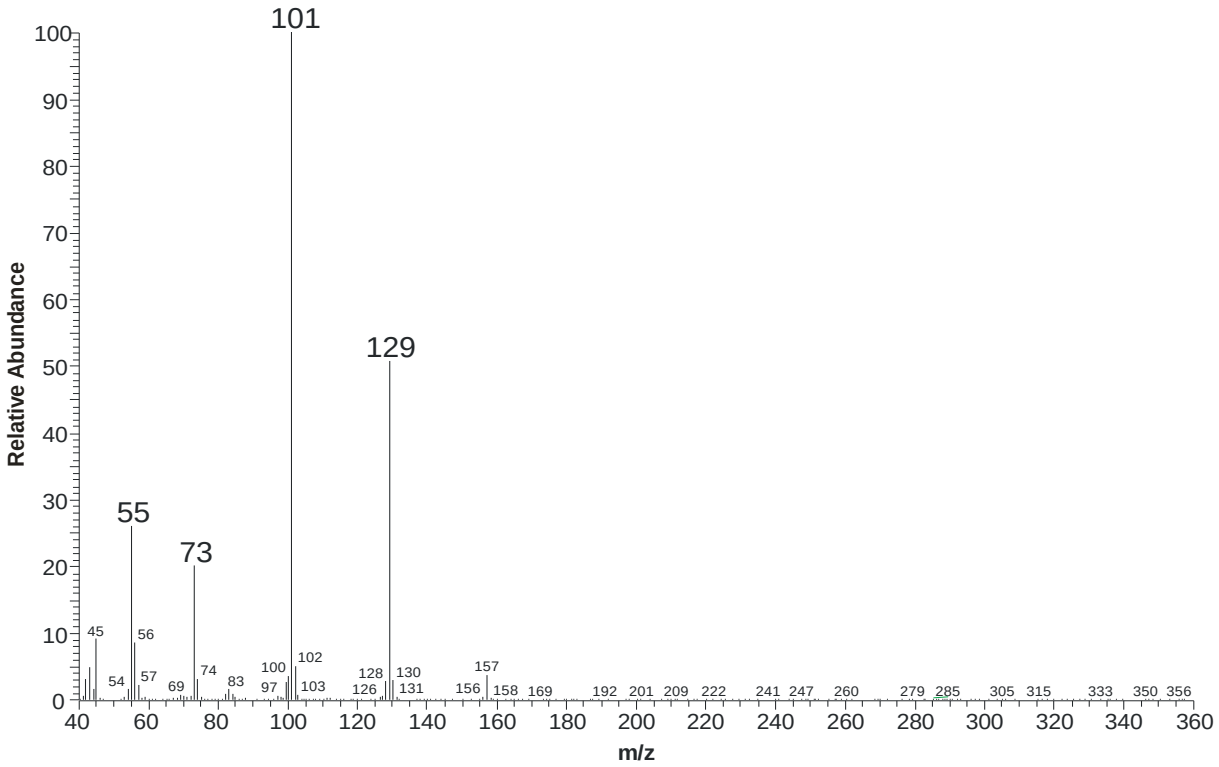



**Fig. 14:** GC-MS analysis, ECF derivatization, EI spectrum – α-ketoglutaric acid (1), the exact structure of the derivative undefined

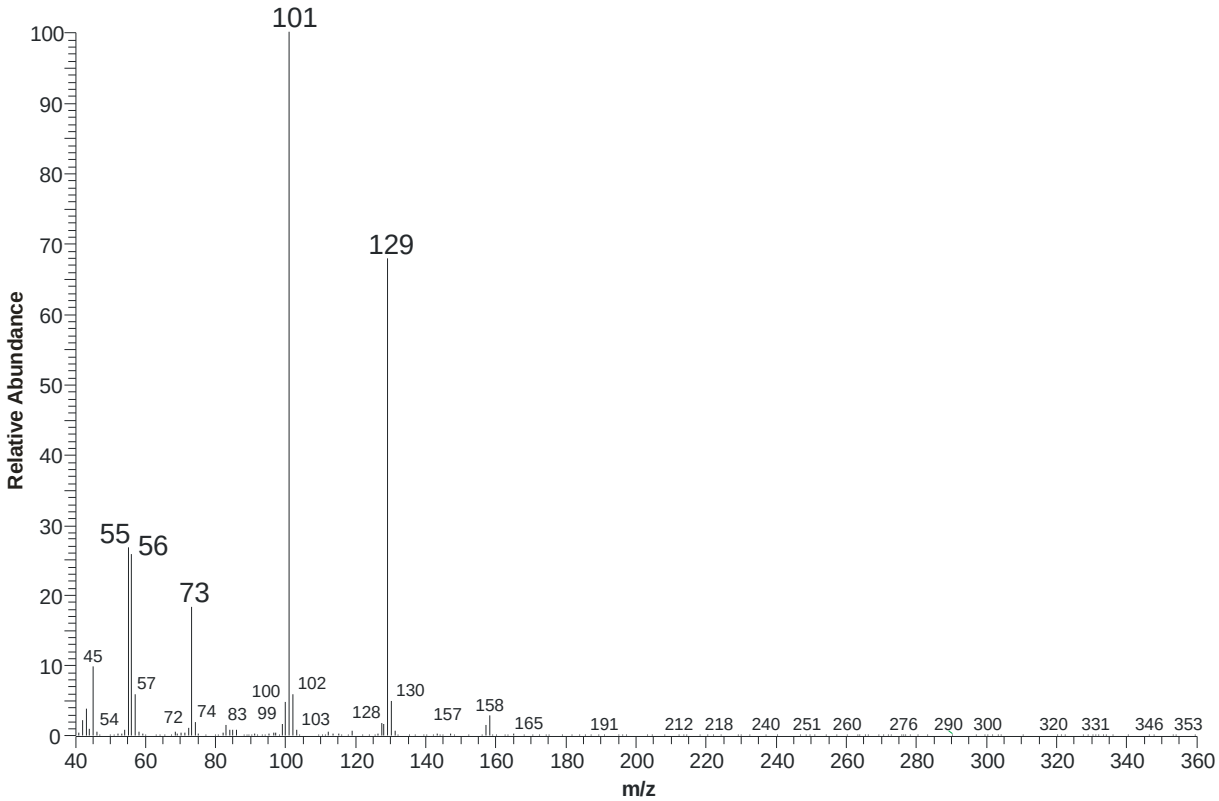



**Fig. 15:** GC-MS analysis, ECF derivatization, EI spectrum – α-ketoglutaric acid (2), the exact structure of the derivative undefined

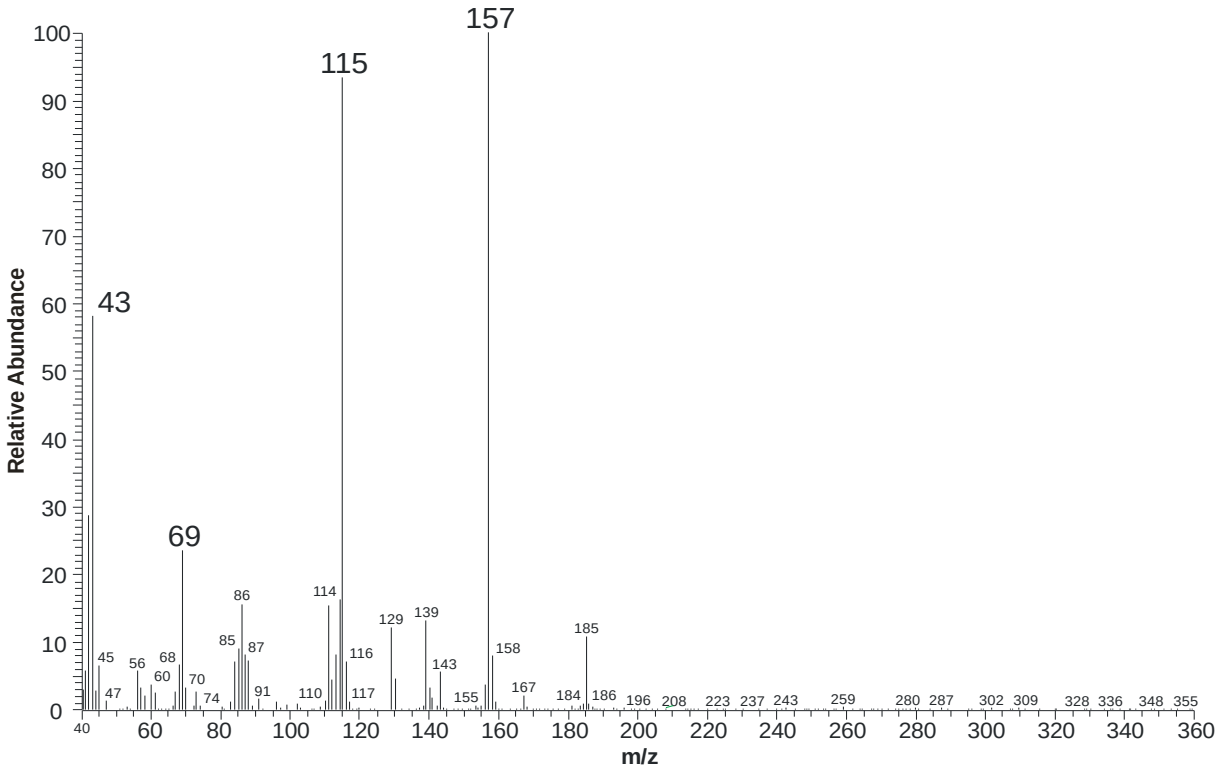



**Fig. 16:** GC-MS analysis, ECF derivatization, EI spectrum – citric acid (1), the exact structure of the derivative undefined

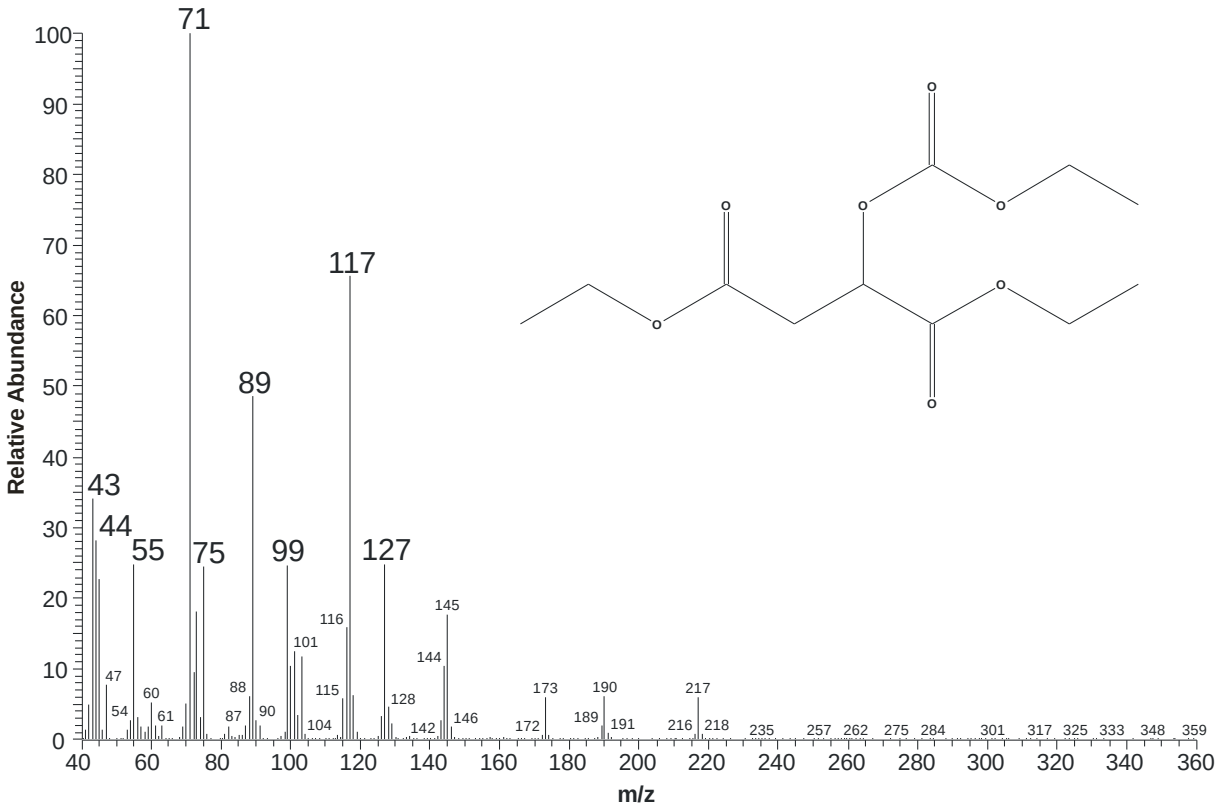



**Fig. 17:** GC-MS analysis, ECF derivatization, EI spectrum – malic acid

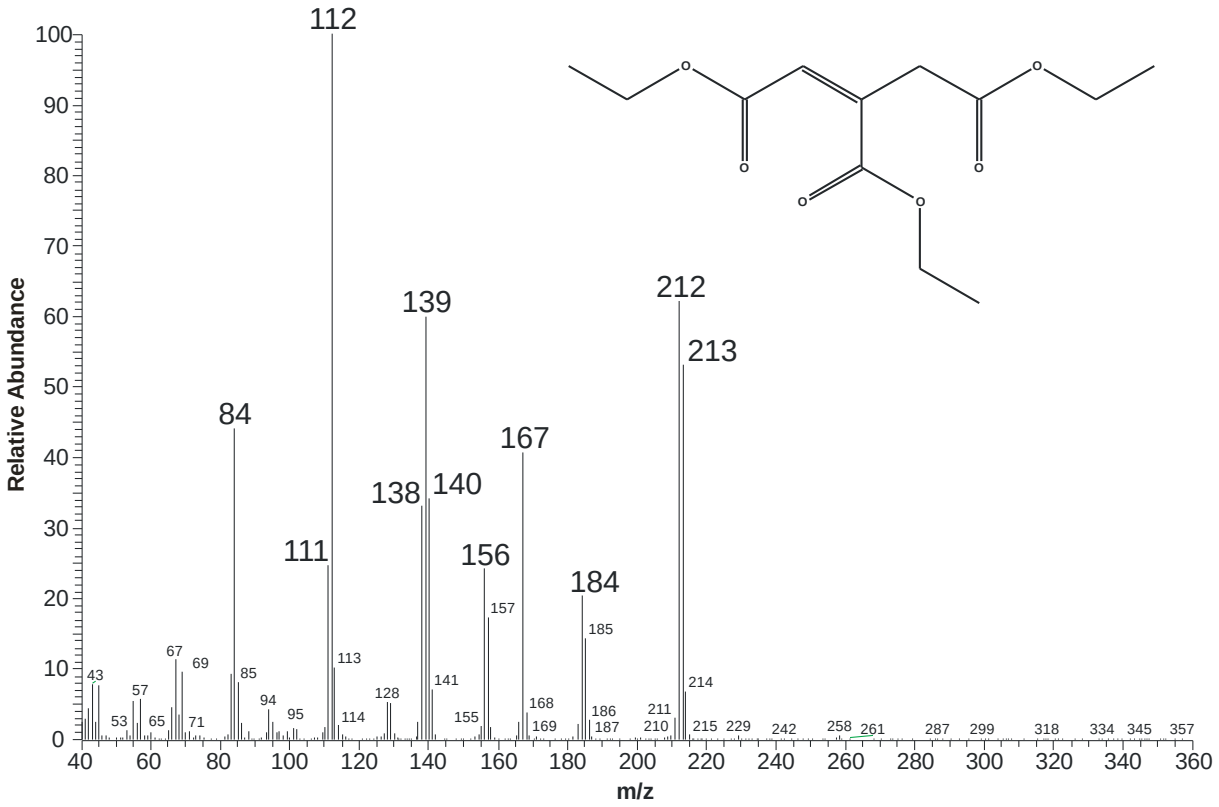



**Fig. 18:** GC-MS analysis, ECF derivatization, EI spectrum – cis-aconitic acid

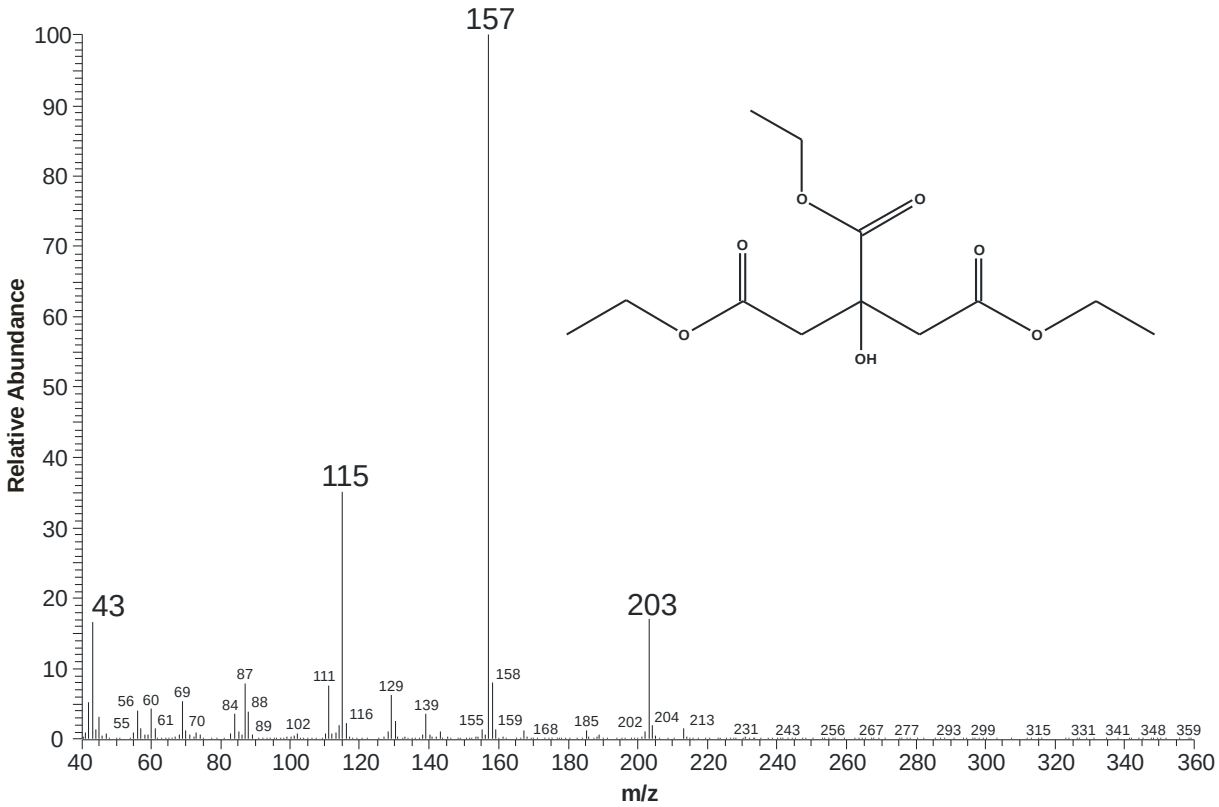



**Fig. 19:** GC-MS analysis, ECF derivatization, EI spectrum – citric acid (2)

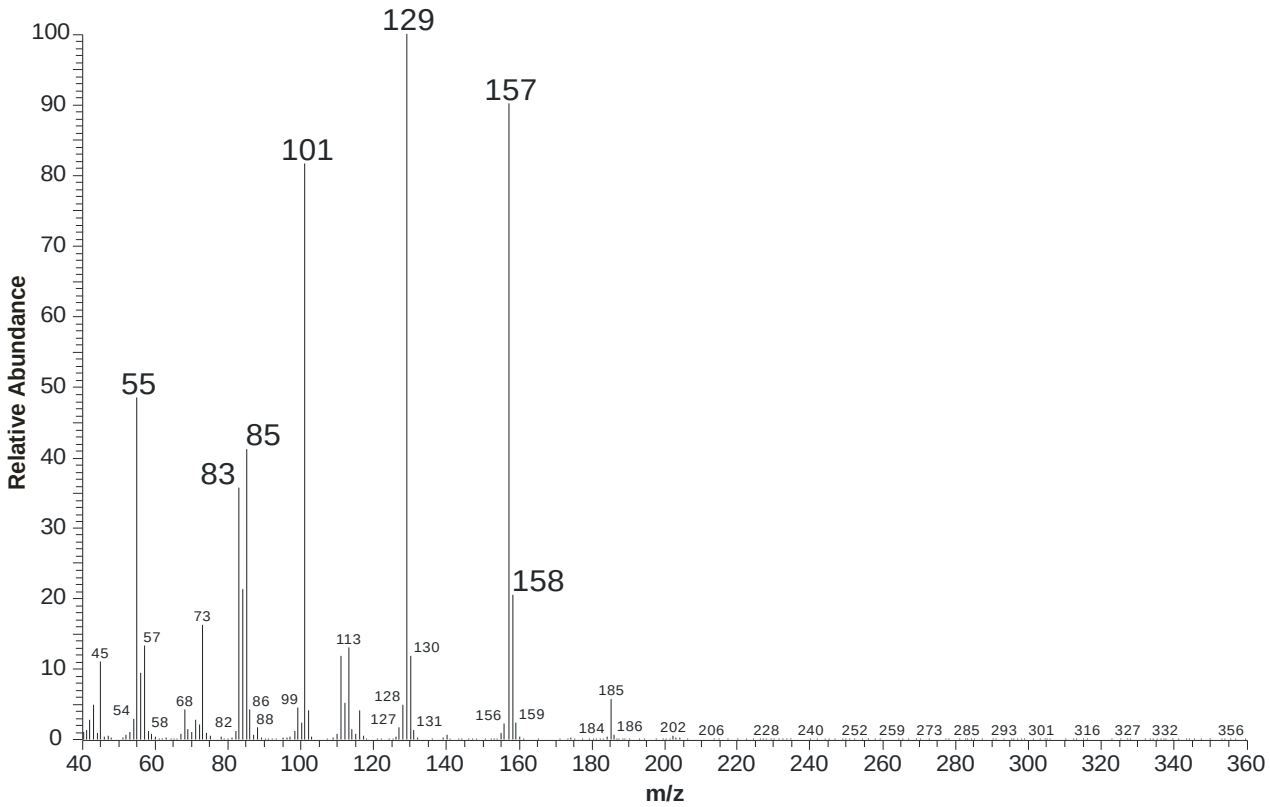



**Fig. 20:** GC-MS analysis, ECF derivatization, EI spectrum – isocitric acid (1), the exact structure of the derivative undefined

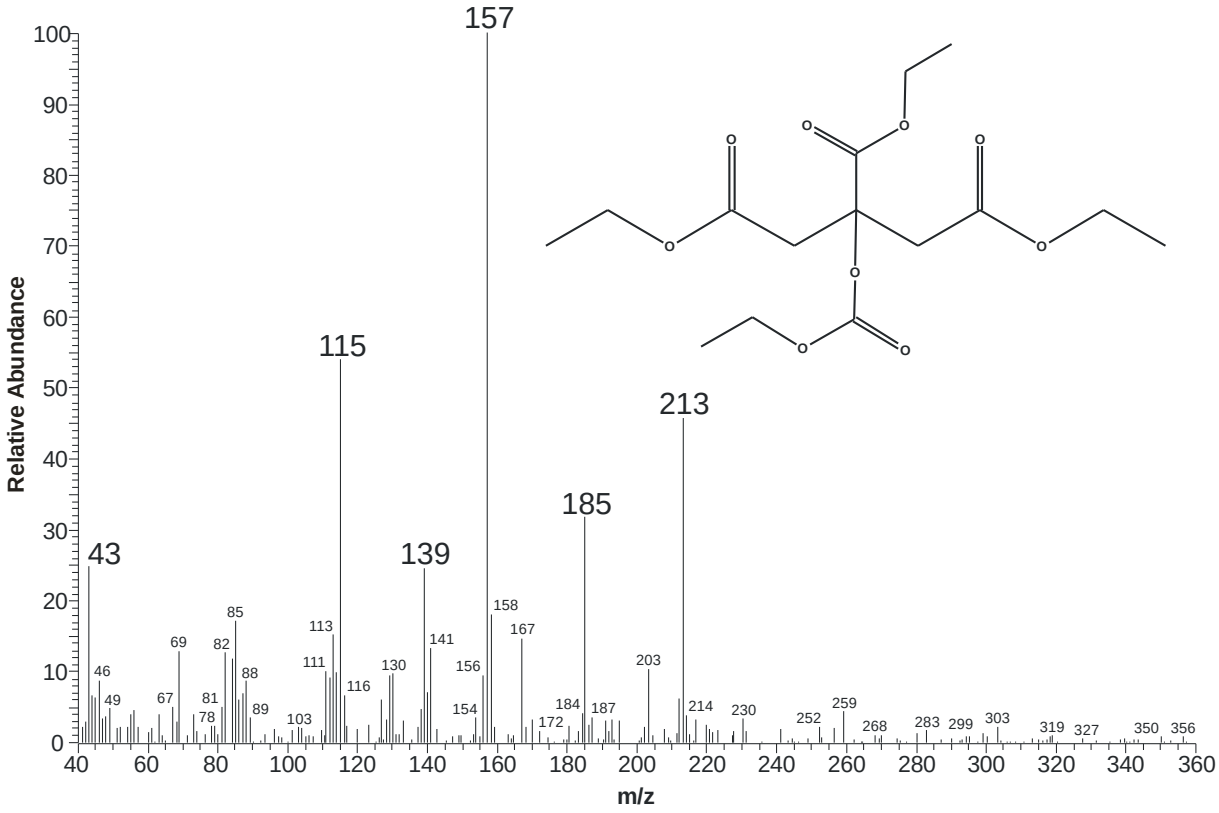



**Fig. 21:** GC-MS analysis, ECF derivatization, EI spectrum – citric acid (3)

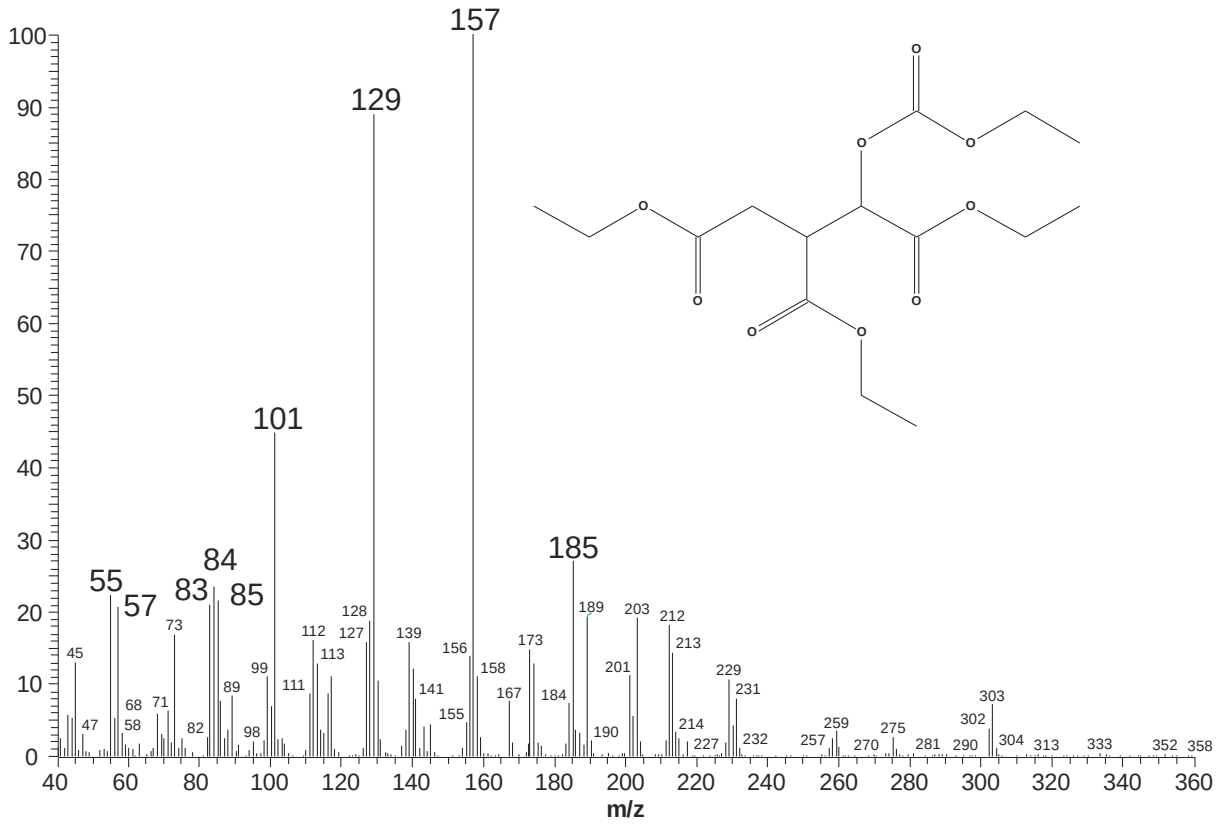



**Fig. 22:** GC-MS analysis, ECF derivatization, EI spectrum – isocitric acid (2)

# 9. Appendix 3: LC-MS analysis – ESI positive spectra of ECF derivatives

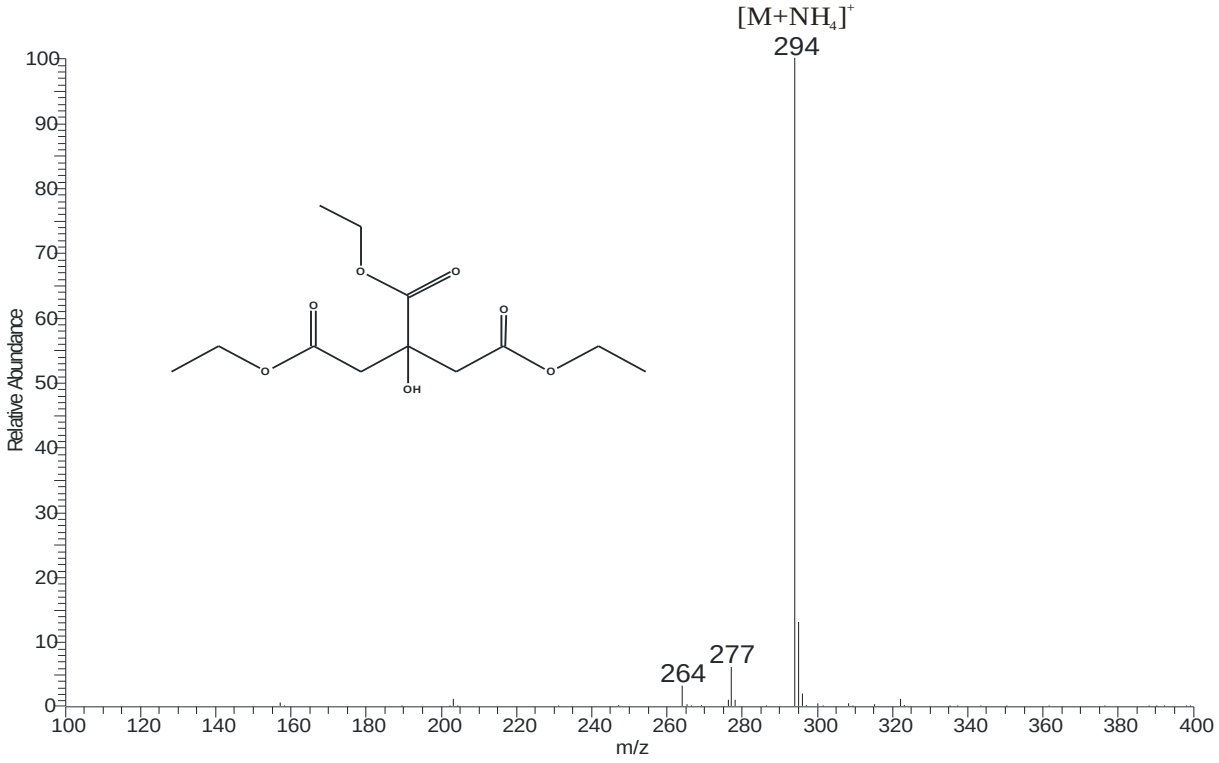



**Fig. 23:** LC-MS analysis, ECF derivatization, ESI positive spectrum – citric acid (1)


**Fig. 24:** LC-MS analysis, ECF derivatization, ESI positive spectrum – malic acid

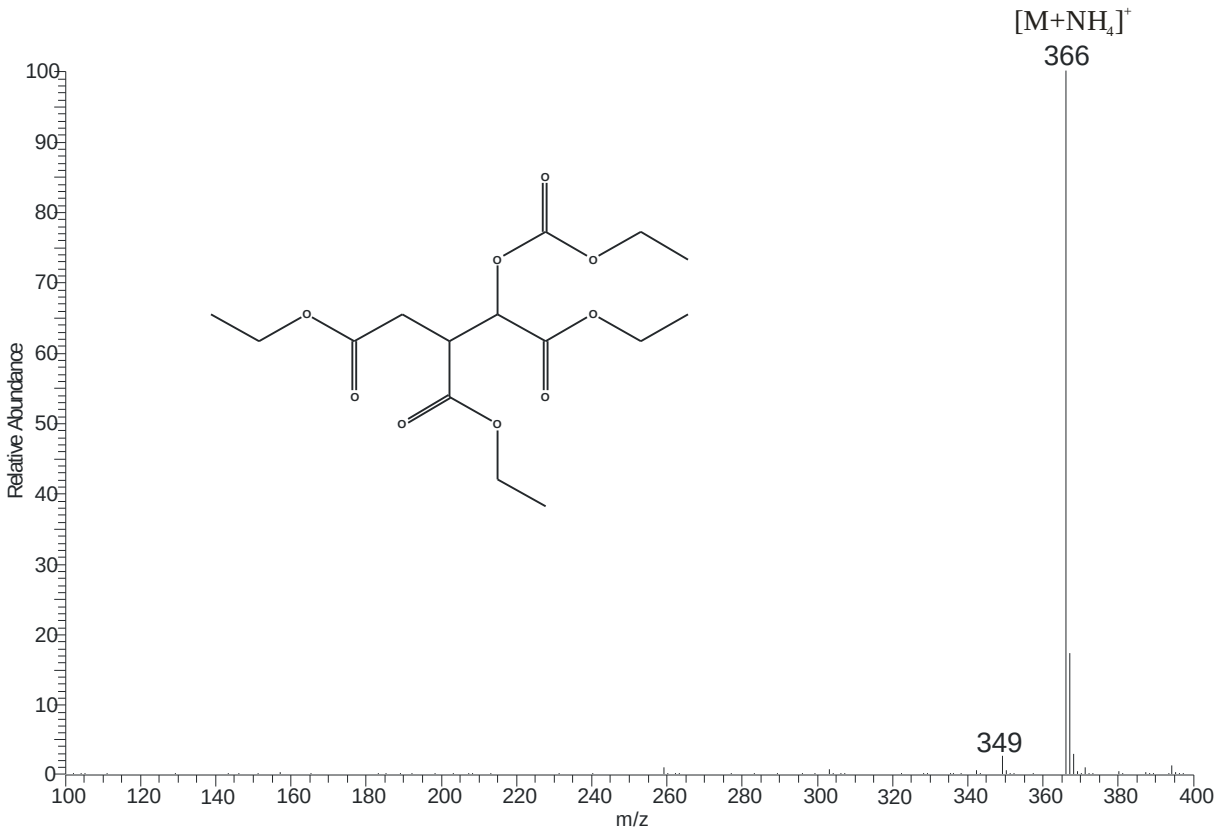



**Fig. 25:** LC-MS analysis, ECF derivatization, ESI positive spectrum – isocitric acid

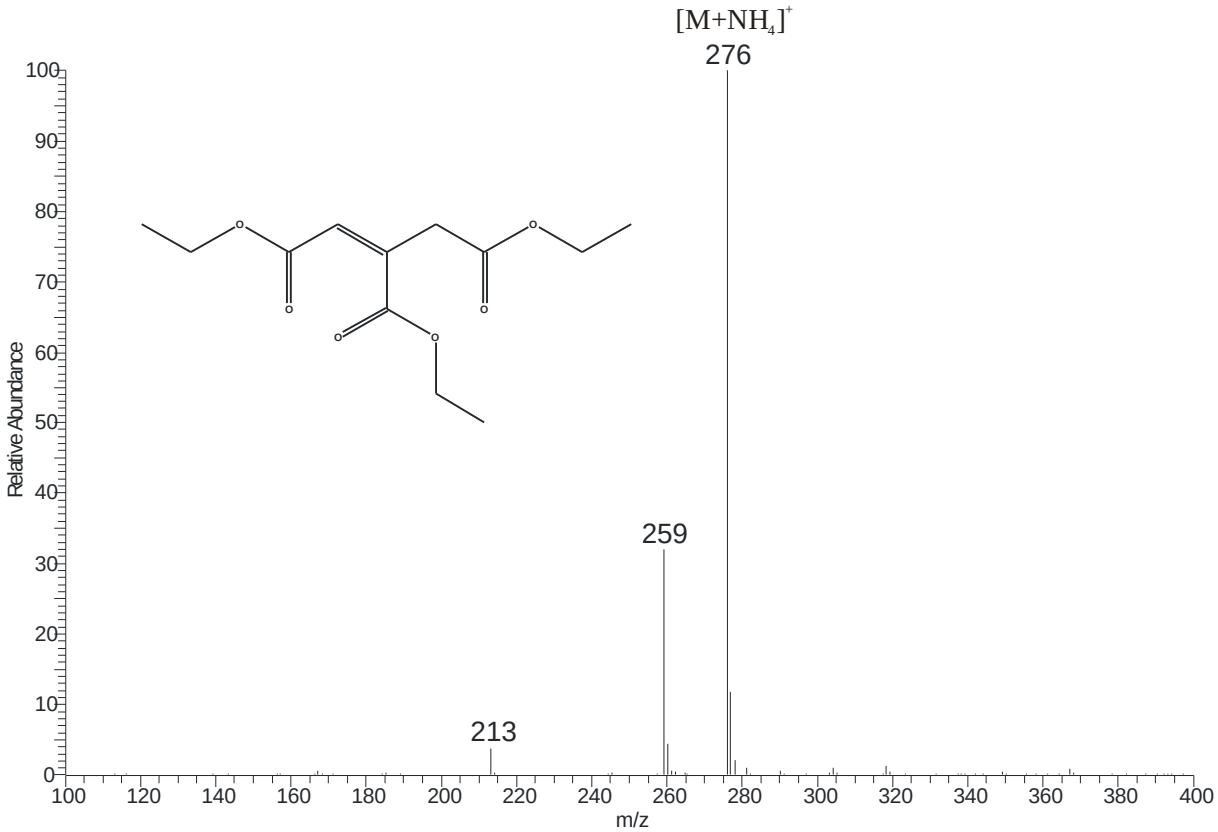



**Fig. 26:** LC-MS analysis, ECF derivatization, ESI positive spectrum – cis-aconitic acid (1)

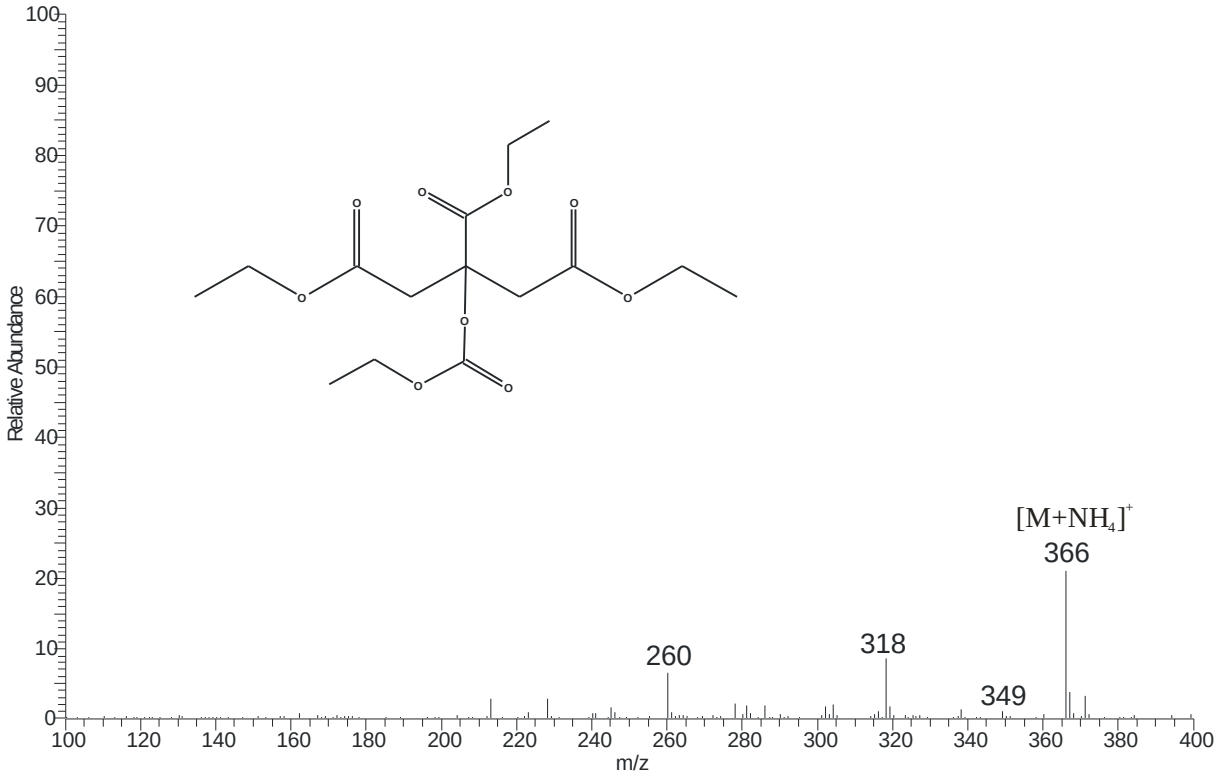



**Fig. 27:** LC-MS analysis, ECF derivatization, ESI positive spectrum – citric acid (2)