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Method Validation of Short Chain Carboxylic Acids

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Abstract

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This thesis project was done in Research Analytics Team at the Chemistry Department of Neste Oil Research and Development. The aim of the study was to validate an existing analysis method in a new bio-oil based sample matrix called brown grease. The analysis method was used to detect short chain carboxylic acids from water and bio-oil based samples using LC-MS and LC-UV techniques after the liquid-liquid extraction. Analytes were quantified using internal standards.

The instrument used in this validation was Shimadzu LCMS-2020 which combined liquid chromatography and mass spectrometry. The liquid chromatography separation method used was ion exclusion chromatography, which was coupled to both MS and diode array detectors. The compounds were ionized using electrospray ionization in negative mode prior to the MS detection.

In this validation, limit of detection (LOD), the limit of quantification (LOQ) and the method accuracy of analytes were determined. The method accuracy was determined using precision and trueness. Samples were prepared in three spiking levels, and they were analysed 6–9 times including the non-spiking samples over 2–4 weeks.

The limit set for precision was 25 per cent and for trueness 80–120 per cent. For LOD and LOQ there were no set limits. Most of the results were within these parameters and as result the sample matrix can be analysed using the existing method for sample preparation and analysing.

Keywords: Short chain carboxylic acids, LC-MS, validation

Tiivistelmä

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Opinnäytetyön tarkoitus oli validoida jo käytössä oleva menetelmä uudelle näytemateriaalille. Menetelmällä analysoitiin lyhytketjuisia karboksyylihappoja bioöljy- ja vesipohjaisista näytteistä, ja uusi näytemateriaali olikin bioöljyä. Työ toteutettiin Neste Oilin Tutkimus- ja kehitysosaston kemian osastolla Tutkimusanalytiikan ryhmässä.

Analysaattorina oli Shimadzun LCMS-2020, jossa menetelmänä toimi nestekromatografian ja massaspektrometrin yhdistelmä. Esikäsittely tehtiin neste-nesteuutolla. Näytteiden yhdisteet erotettiin ioniekskluusiokromatografialla, ionisoitiin sähkösumutuksella ja detektorina toimi massaspektrometri. Yhdisteiden kvantitointi tehtiin käyttämällä sisäisen standardin menetelmää.

Validoinnissa määritettiin menetelmän tarkkuus sekä määritys- ja havaitsemisrajat. Tarkkuutta arvioitiin laskemalla täsmällisyys sekä oikeellisuus. Näytteestä tehtiin kolme eri tasoa lisäämällä analyyttiliuosta. Nämä sekä näyte ilman lisäyksiä analysoitiin 6–9 kertaa 2–4 viikon aikana.

Täsmällisyydelle asetettiin hyväksymisrajaksi 25 prosenttia ja oikeellisuudelle 80–120 prosenttia. LOD- ja LOQ-arvoille ei ollut hyväksymisrajoja. Enimmäkseen tulokset olivat annettujen rajojen sisällä, joten validointia voidaan sanoa onnistuneeksi.

Avainsanat: Lyhytketjuiset karboksyylihapot, LC-MS, validointi

Contents

List of Abbreviations

ACN	Acetonitrile
APCI	Atmospheric Pressure Chemical Ionization
API	Atmospheric Pressure Ionization
CRM	Certified Reference Material
DAD	Diode Array Detector
EI	Electron Impact Ionization
ESI	Electrospray ionization
HILIC	Hydrophilic Interaction Chromatography
HMF	Hydroxymethylfurfural
HPLC	High Performance Liquid Chromatography
HPLC-MS	High Performance Liquid Chromatography – Mass Spectrometry
IEC	Ion Exclusion Chromatography
ISTD	Internal Standard
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification

m/z	Mass-to-charge ratio
MALDI	Matrix Assisted Laser Desorption Ionization
MCE	Mixed Cellulose Esters
MS	Mass Spectrometry
RSD	Relative Standard Deviation
STD	Standard Deviation

1 Introduction

As the world's need for oil and especially for renewable energy grows, more and more impure source materials are used to meet this demand. These materials can contain varying amounts of compounds that may affect the reprocessing procedure. For this reason, the materials need to be analysed before production. One of these new materials is a bio-oil based waste that comes from food processing facilities such as dairy industries, and this is the sample matrix used in this work. (Jin 2021a.)

This thesis project was conducted in Research Analytics Team at the Chemistry Department of Neste Oil Research and Development in Porvoo Finland. The purpose was to validate a method for the determination of short chain carboxylic acids from bio-oil based sample matrix using LC-MS. The method for analysis and sample preparation was already in use but the sample matrix was new. (Jin 2021a.)

As the method was already in use, the validation was limited to only few parameters. Those were precision, limit of detection (LOD), limit of quantification (LOQ), and trueness. (Jin 2021a.)

The project was done with the supervision of Neste Oil researcher Chunfen Jin and all the decisions about the analyses and validation were done with her guidance.

2 Carboxylic Acids

Short chain carboxylic acids are organic compounds that have chains of one to six charcoals and have the carboxyl functional group (-COOH) attached to it. These are very polar compounds that can easily either receive or give a hydrogen atom and therefore participate in hydrogen bonding. They are typically weak acids and dissociate only partly in aqueous solutions but are more soluble in non-

polar solvents. (Hänninen, Karppinen, Leskelä & Pohjakallio 2019, 193-202.) The structure of carboxylic acid can be seen in figure 1.

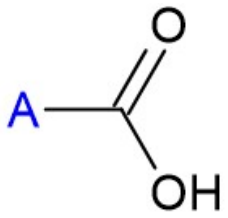

Figure 1. Carboxylic acid.

These short chain carboxylic acids are one of the most important impurities found in raw materials used in production of renewable energy. Because they are also corrosive and some of them volatile, the quantity of these acids needs to be determined before they can be used in such production. (Viidanoja 2015.)

3 Furfural And Hydroxymethylfurfural

Furfural and hydroxymethylfurfural (HMF) are both organic compounds including a furan ring and an aldehyde functional group (-CHO) attached to the ring as can be seen in figure 2 (Furfural 2005).

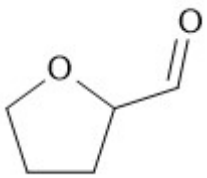

Figure 2. Furfural.

In addition to furfurals furan ring and aldehyde functional group, HMF also has an alcohol functional group (-OH) as seen in figure 3 (5-Hydroxymethylfurfural 2005).

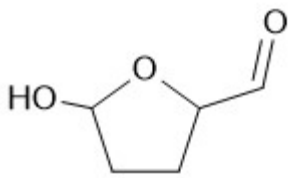

Figure 3. Hydroxymethylfurfural.

Both furfural and HMF are furans as they have the furan ring, and they are formed when carbohydrates decomposes as they are exposed to high temperatures. For this reason, these furans can be found in many food processing products and waste. These, however, are not corrosive or harmful in production but actually good source materials for fuel and chemical production and are analysed for that reason. (Van Putten, van der Waal, de Jong, Rasrendra, Heeres, & de Vries 2013; Ebert 2021.)

3.1 Analysing Methods for Carboxylic Acids in Oil

Short chain carboxylic acids have been analysed using ion chromatography (IC), gas chromatography (GC), and high performance liquid chromatography (HPLC) but these all have some issues with accuracy or reliable quantification. IC often suffers from contamination in the column because the oil matrices have components with low solubility. This would contaminate further analyses as well or make the cost of the analysis high with shortened column replacement schedules. GC needs the sample to be derivatized if accurate and highly sensitive results are wanted. The derivatization makes them volatile and sample loss can occur in split injection. Using HPLC without mass spectrometer does not give reliable quantitative results with such complex sample matrix as oil. Derivatization might help this, but just as in GC the derivatized forms are partly soluble in water and sample loss during sample preparation may occur. This recovery lost can be corrected by using the internal standard method. Viidanoja conducted a study in 2015 focusing on liquid chromatography attached with mass spectrometer. This yielded good results and the method used in this study was defined according to those results. (Viidanoja 2015.)

4 Liquid-liquid Extraction

Liquid-liquid extraction is a separation method. The extraction is done by adding a liquid solvent to a liquid that contains the target compounds. The liquids used in this extract method must be such that they will not blend. These will be separated using either a separating funnel or, as in this thesis, a centrifuge. The different solvents are chosen depending on the attributes of the target compounds. For extracting grease and other non-polar compounds, it is common to use hydrocarbons or chloride hydrocarbons. For polar compounds good solvents are water and alcohols. (Eskeli, Hamara, Laukkanen, Lehtonen, Vihavainen & Ylihärsilä 2021.) In this thesis the solvents used were chloroform and water. This means the carboxylic acids were separated to the upper water layer and oils and other not wanted compounds were separated to the lower chloroform layer.

5 Internal Standard Method

In internal standard (ISTD) method a known quantity of a standard is added to the sample before sample preparation. This standard resembles the target analyte and behaves the same way during the preparation. This counts in possible recovery loss during the preparation as the quantity of the standard is known. This way any recovery loss observed of the standard can be used to calculate recovery loss of the sample. For this reason, internal standards are commonly used especially when the analysis includes complex sample preparation or when the effects of the sample matrix to the signal cannot be eliminated. This way the ratio between the standard and the analyte should remain the same as the same fraction of both is loss during preparation. (Jaarinen & Niiranen 2018, 22.)

When calibrating the analyser, the internal standard is added into all the calibration levels. Knowing the concentrations of both the standard and the calibration levels the calibration curve can be calculated. This is done with the

ratios between the concentrations and the ratios between the signals. (Jaarinen & Niiranen 2018, 22.) To count the concentrations in samples a response factor must be calculated. This is the ratio between the signal of the target analyte in the sample to the signal of the internal standard in the sample (Harris 2007, 90). This is shown in equation 1.

$$F = \frac{A_A}{C_A} / \frac{A_{istd}}{C_{istd}} \quad (1)$$

where

A_A = area of the analyte
C_A = concentration of the analyte
A_{istd} = area of the internal standard
C_{istd} = concentration of the internal standard.

The analyte concentrations from samples are calculated using equation 2 when using linear calibration curve and equation 3 when the calibration curve is based on quadratic equation (Nousiainen 2015, 18–19).

$$\frac{Area_A}{Area_{istd}} = F * \frac{[A]}{[istd]} + b \quad (2)$$

where

$Area_A$ = the area of the analyte spike
$Area_{istd}$ = the area of the internal standard spike
[A] = the concentration of the analyte
[istd] = the concentration of the internal standard
b = constant.

$$\frac{Area_A}{Area_{istd}} = a * (\frac{[A]}{[istd]})^2 + b * \frac{[A]}{[istd]} + c \quad (3)$$

where

$Area_A$ = the area of the analyte spike
$Area_{istd}$ = the area of the internal standard spike
[A] = the concentration of the analyte
[istd] = the concentration of the internal standard
a = response factor
b = response factor
c = constant.

6 High Performance Liquid Chromatography

All chromatography is based on balances between a mobile and a stationary phase, and in liquid chromatography (LC) the mobile phase is in liquid form. The sample molecules or ions are dissolved in this mobile phase. (Jaarinen & Niiranen 2018, 140.)

High performance liquid chromatography is a separating method that can be used for a great number of different compounds even at the same time. The requirement for the sample is merely that it can be dissolved in liquid eluent. For this reason, HPLC can be used for separating inorganic as well as organic molecules and ions. It is also extremely versatile when it comes to the size of the compounds, and it has no inhibitions for volatility or derivatives. (McMaster 2007, 3.)

First step of liquid chromatography is that the sample dissolved in liquid solvent is injected into the liquid eluent that is the mobile phase and pumped through thin capillaries under high pressure. The eluent must run smoothly without pulsating to keep the conditions steady. This requires consistency from the pumps. The separation then occurs when the mobile phase passes through a column which is packed with the stationary phase. The stationary phase is comprised of small particles with which compounds in the sample will interact. The interaction depends on the ion-exchange, adsorption, partitioning, or size. The compounds

in the sample will attach to the stationary phase and detach from it to the mobile phase. The compounds' balance between the two phases varies and this separates them, and they all arrive at different times to the detector. (McMaster 2007, 4–5.)

6.1 Ion Exclusion Chromatography

Ion exclusion chromatography (IEC) is a form of chromatography where ions are separated according to their charge. This is done by creating a charge to stationary phase. This means that negatively charged ions attach themselves to a positively charged phase and vice versa. This excludes the ions with opposite charge quickly as they cannot attach. This way the ions move through the column with the speed depending on their electricity level. As the stationary phase is made from porous material, larger ions are also excluded as they cannot penetrate the small pores. (Lodi, Storti, Pellegrini & Morbidell 2017; Harris 2007, 593.)

6.2 Diode Array Detector

Diode array detector (DAD) is one of the detection methods used in liquid chromatography. It consists of photodiodes that are arranged linearly. The light is directed to the diodes through a diffraction grate. Typically, the number of diodes used is either 512 or 1024. The diodes measure the absorbance of light separately on different wavelengths. Depending on the detector's wavelength range as well as the number of the diodes in the array, each diode collects data from 0.5 nm to 1 nm range. This makes it possible to measure wavelengths from 190 nm to 800 nm at the same time without the wavelengths changing between the measurements. This requires one lamp for ultraviolet light and another for visible light. (Dolan 2016.)

7 Mass Spectrometry

Mass spectrometry is an analytical method for analysing inorganic and organic compounds. First the compounds are ionized and then they are separated according to their mass-to-charge ratio (m/z). After the separation, the compounds can be measured quantitatively or qualitatively by the intensity of their m/z. The compounds can be ionised by several different methods. The most common ones are electron impact ionization (EI), atmospheric pressure chemical ionization (APCI), matrix assisted laser desorption ionization (MALDI) and the one used in this study electrospray ionization (ESI). (Ketola, Kostiainen, Kotiaho & Vainiotalo 2010, 15.)

Mass spectrometer consists of injector, ionization, mass analyser, detector, vacuum pumps, and a computer that controls the components as well as collects and analyses the measured data. The mass analyser and detector are kept in vacuum so no ions and surrounding gas molecules would collide. Some ion sources can use vacuum as well. (Ketola etc. 2010, 15.)

7.1 Electrospray Ionization

Electrospray ionization is an ionization method in mass spectrometry where liquid is transformed into an aerosol using a high voltage. An inert gas can also be applied to enhance the ionization. Most used gas is nitrogen. This method differs from the other ionization methods because ESI can produce multiple charged ions. It also causes minimal fragmentation which can be an issue in some other ionization methods. (Ketola etc. 2010, 71.)

An ESI ion source consists of thin steel capillary and an atmospheric pression ionization (API) -source. The capillary's inner diameter is usually between 0.1 and 0.2 mm wide. When using a gas-assisted ESI, the steel capillary is placed inside another capillary and a gas flow runs between them. The gas flow causes a turbulence at the end of the capillary which enhances the formation of the aerosol. (Ketola etc. 2010, 71–72.)

In ESI the sample is dissolved into a liquid eluent which is then injected through the steel capillary using 2–10 µl/min flow rate. This capillary has a high voltage coming from the ion source. This forms a high electrostatic field at the end of the capillary. This repels the ions with opposite charge of the voltage conducted from the ion source to the capillary walls. Because of the electrostatic field the target ions drift onto the surface of the eluent. This makes the surface flow faster than the center of the liquid and causes a funnel shaped phenomena called Taylor cone. As the eluent flow narrows the repulsion of the charges increases and charged droplets are formed. These will then break into even smaller droplets as solvent is evaporated and the charge density increases. This happens multiple times until the droplets are small enough for gas phase. These gas ions are then collected using the API-source and directed to the mass spectrometer. (Ketola etc. 2010, 71–72.) The schematics of the ESI is presented in figure 5.

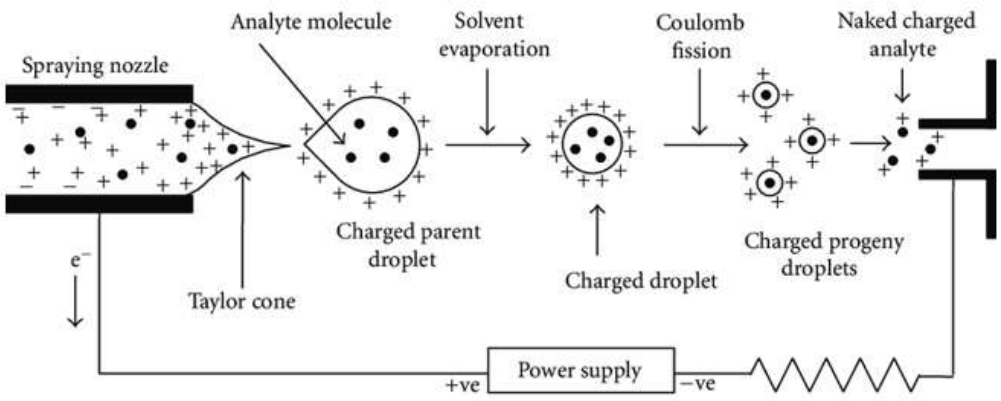

Figure 4. Electrospray Ionization (Banerjee 2012, 7).

7.2 Quadrupole Mass Analyser

Quadrupoles are filters that let ions pass according to their m/z ratio. A quadrupole has four parallel metallic electrodes which are called quadrupole rods. The voltage in these rods forms an electric field between them. An ideal quadrupole rod should be hyperbolic but as it is hard to manufacture the usual shape is round. (Ketola etc. 2010, 27.)

The filtration starts as the ions arrive between the quadrupole rods. The electric field between the rods forces the ions to move in a vibratory motion that is perpendicular to the direction they are moving. If the vibration of the ion is too wide, they will collide with the rods. The voltage of the rods is set so that they will only let pass ions with the right m/z ratio. This way only the targeted ions are directed to the detector. (Ketola etc. 2010, 28.)

7.3 Electron Multiplier Detector

Electron multiplier enhances ion flow by emitting electrodes their quantity increasing from dynode to dynode. As positively charged ions hits the first dynode multiple electrodes and negatively charged ions are detached. These are flown to the next dynode by the difference in potential between the dynodes. Their collision to the next dynode attaches again more electrodes. This goes on for 10–20 times until per every ion there is 10^5 electrons. These arrive to the last dynode called collector which has zero potential and detects the electron flow. For negative ions, the first dynode has high positive voltage, and it releases positive ions. This is called conversion dynode. After this first conversion the approach is the same as above. (Ketola etc. 2010, 97.)

7.4 Validation

Validation is a procedure where an analysing method is tested and proven to be suitable for the intended use. The performance of the method is tested with various measurements. The data received from the analyses is collected and the reliability of the method is then determined according to this and possible previous information about the subject. (Jaarinen & Niiranen 2018, 11.)

The chosen measurements depend on the analysing method, on the sample matrix, and on the number of the measured analytes. There are a lot of parameters to calculate from the received data. In this study these parameters were limited to limit of detection, limit of quantification, and accuracy which was calculated using precision and trueness. This was because the method was

already in use and the aim was to validate a new sample matrix for that existing method. (Jaarinen & Niiranen 2018, 11.)

7.4.1 Reference Sample

Reference sample is a form of quality control to make sure the analyser is performing as intended. It is used when a certified reference material (CRM) either is not available, or it cannot be used for reasons like unstable sample materials. The reference sample consists of the same kind of matrix as actual samples. It must also include concentrations of target analytes that are known precisely. This sample should be analysed regularly depending on the frequency of the analyses. The reference sample should give similar results with every analysis. Usually, the limits are set inside 2*standard deviation (std) from the mean after analysing the sample first at least 20 times. If gotten results do not match known concentrations, the analysis cannot be considered reliable. This means the analyser might need maintenance or the calibration renewing. (Ambrus, Cortes-Toro, El Bidaoui, Fajgelj, Gardiner, Ihnat, Chuanfan & Rossbach 2003, 7.)

7.4.2 Accuracy

Accuracy is used to describe how well a single measured value corresponds to a reference value. In validation the accuracy is determined by evaluating systematic as well random effects on results. To count in both effects, accuracy must be examined as two factors. These are precision and trueness. (Magnusson & Örnemark 2014, 31.)

Precision tells the fluctuation between analyses by expressing how close results are to each other. For this there needs to be done several measurements separately in different conditions. This tells then how similar the results are with each other regardless of time and date. (Jaarinen & Niiranen 2018, 12; Magnusson & Örnemark 2014 2014, 35.) Precision is presented using relative

standard deviation (RSD-%). It is calculated from the standard deviation (s) and mean ($\bar{x}$) of the results using equation 4.

$$RSD = \frac{S}{\bar{x}} * 100\ \% \quad (4)$$

Trueness is the compatibility between a measured value and expected value. It measures how close the mean of the results is to a reference value. This is impacted by systematic error. Trueness is expressed with 95 per cent confidence level. (Jaarinen & Niiranen 2018, 12; Magnusson & Örnemark 2014, 31.) Trueness can be expressed using certified reference material or as in this thesis with recovery–%, which is calculated using the equation 5.

$$Recovery - \% = \frac{C_{spiked} - C_{non-spike}}{C_{solution}} * 100\ \% \quad (5)$$

where

C_{spiked} = the concentration of the spiked sample
$C_{non\text{-}spiked}$ = the concentration of the non-spiked sample
$C_{solution}$ = the concentration of the spiking solution.

7.4.3 Limit of Detection

Limit of detection is the concentration of the analyte that can be reliably detected within the chosen confidence level. This can be determined by analysing a sample with low concentration multiple times. For this the sample must be prepared separately for every analysis. Determined value must differ significantly from the value of the zero sample. (Jaarinen & Niiranen 2018, 13.) LOD can be determined by the standard deviation of the low concentration sample or as was done in this thesis by the signal-to-noise-ratio (S/N). The LOD is the concentration where the S/N-ratio is between 3 and 2:1. (Jin 2021a.) S/N-ratio can be seen directly from the analyser.

7.4.4 Limit of Quantification

Limit of quantification is the concentration of the analyte that can be reliably quantified within the chosen confidence level. (Jaarinen & Niiranen 2018, 13.) This limit can be calculated as LOD using the standard deviation of the low concentration sample or estimating it using either RSD or S/N-ratio. The latter was used in this thesis. This way the limit is set at the concentration where the S/N-ratio is ≥10. (Jin 2021a.)

When a sample concentration is between the values of LOD and LOQ that analyte can be determined to be in the sample, but the value cannot be reliably quantified. For these instances, the concentration is commonly answered as under the value of LOQ. (Jaarinen & Niiranen 2018, 13.)

8 Chemicals And Equipment

A lot of different solvents and compounds both in liquid and solid form were used during the laboratory work. Every solvent was LC-MS-grade and did not need to be filtered before use. The analysing equipment is specified in chapter 3.3 but in addition to that during sample and solution preparation a lot of basic laboratory equipment was used. The eluents were made in graduating glass bottles using measuring glasses and the stock solutions either in volumetric flasks or in 4 ml deactivated glass vials. Samples were initially weighed in 2 ml Eppendorf tubes and after extraction they were filtered using sterile 5 ml syringes and 0.45 µm mixed cellulose esters (MCE) membrane filters to 1.2 ml glass vials. During sample, eluent, and analyte solution preparation a scale and a Vortex mixer as well as volumetric and Pasteur pipettes were used constantly. The scale was inspected weekly, and the volumetric pipettes were calibrated in an accredited service before starting the second phase of the validation.

8.1 Reagents

8.1.1 Eluents And Sample Preparation

There were three different eluents that were prepared for the analysis. These are described in table 1. These eluents were made using water, ACN, hydrochloric acid (HCl), 2-propanol and 25 % ammonia-water-solution.

Table 1. Reagents used in eluents.

	Eluent A	Eluent B	Eluent C
Water (vol-%)	100	70	-
ACN (vol-%)	-	30	-
30 % HCl (µl)	625	625	-
25 % ammonia-water solution (vol-%)	-	-	0,2
2-propanol (vol-%)	-	-	99,8

The first one used in the analysing process was eluent A and the second one was eluent B. This second one had ACN added to it to aid the hydrophobic components eluting from the column before injecting the sample to the ion source. This was also used to remove hydrophobic impurities from the column at the end of the analysis. The third and last eluent, eluent C was directed straight to the mass spectrometer through a T-join.

In addition to these eluents the most used solvents were the two used in sample preparation: chloroform and water.

8.1.2 Standards And Analyte Solutions

The analyte stock solutions were made separately for each compound measured with this method. For these crude products with 100 per cent purity were used

and weighed as shown in Appendix 1. The analyte working solution was made from these stock solutions diluting with eluent A. The preparation is described in chapter 4.3.1.

The internal standard solution was done similarly as the analyte working solution first weighing crude compounds and dissolving them either in water or in 1:1-solution of water and ACN and then pipetting gravimetrically from those stock solutions into one working solution. This was then diluted with eluent A like the analyte working solution. In this ISTD-solution there were only eight compounds in contrast to the analyte working solution that included all the 25 compounds. These compounds in the ISTD were acetic acid, propionic acid, butyric acid, pentanoic acid, hexanoic acid, levulinic acid, succinic acid, and lactic acid. The lactic acid was used in sodium lactate form.

8.2 Analyser

The instrument used in this study was Shimadzu LCMS-2020. This is shown in figure 6. The analyser separated the compounds using ion exclusion chromatography and detected them with quadrupole mass spectrometer. Samples were ionized with electrospray and detected with electron multiplier. The chromatograph also had diode array detector that was used detecting HMF and furfural. (Nousiainen 2015, 6.)

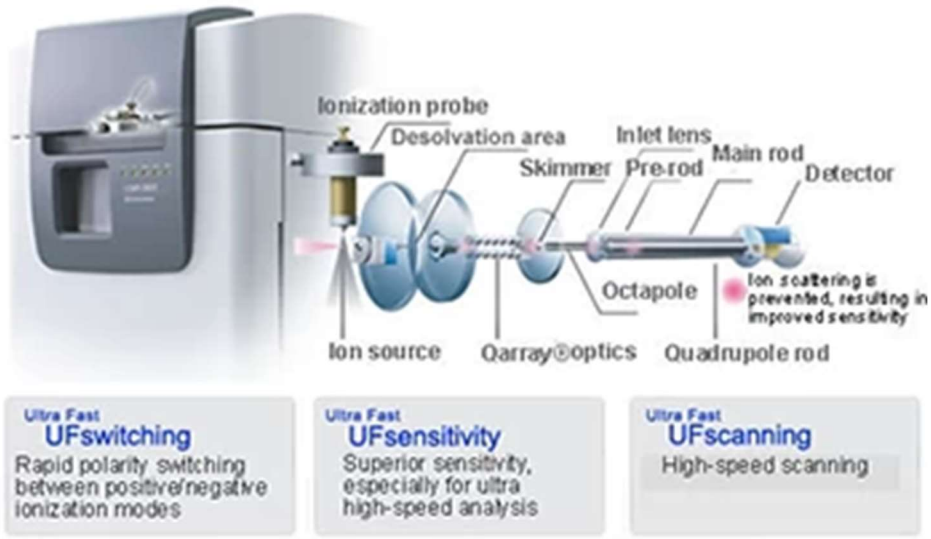

Figure 5. Shimadzu LCMS-2020 Analyser (Shimadzu 2021).

The method was isocratic with the exception that after 32 minutes the flow was changed for 15 minutes to flush impurities from the analytical column. Otherwise, the analysis was performed with pumping eluent A 0.47 ml/min and eluent B 0.15 ml/min but for that 15 minute period eluent A was pumped with 0.06 ml/min flow and eluent B with 0.54 ml/min flow. The eluent C was pumped without change during the whole analysis with 0.06 ml/min flowrate. Eluents A and B were directed through the sampler, but eluent C was directed straight to a T-joint through a 0.010” PEEK tubing. (Nousiainen 2015, 12.)

The eluent carrying the sample were conducted from the analytical column to 1:10 flow splitter through a 0.007” PEEK tubing. From the flow spitter the high flow with 0.54 ml/min flowrate was directed to the DAD and the low flow with 0.06 ml/min flow rate was directed to the T-joint through 0.005” PEEK tubing. The third spot from the T-joint is connected to the ESI source through 0.005” PEEK tubing. (Nousiainen 2015, 12.) Information considering the analyser can be seen in table 2.

Table 2. Analyser information.

HPLC	
Analyser name	Shimadzu Prominance
Degasser	DGU-20A3R
Pumps A & B	LC-20ADXR
Pump C	LC-20AD
Autosampler	SIL-20ACXR (temperature + 4 °C)
Injection volume	50 µl
Column oven	CTO-20AC (temperature + 40 °C)
Security Guards	Phenomenex Carbo-H 4 x 3,0 mm ID SecurityGuard Cartridge
Security Guard Holder	Phenomenex SecurityGuard Cartige kit
Column	Phenomenex rezex ROA-Organic Acid H+ (8 %) 300 x 7,80 mm
Diode Array Detector	SPD-M20A
Flow Spitter	ASI model 620 (split ratio 1:10)
Mass Spectrometer	
Analyser name	Shimadzu MS-2020
Analyser type	Quadrupole
Gas Pressure (N_2)	690–800 kPA
Detector	Electron Multiplier
Ion Source	ESI and APCI
Ionization mode	ESI negative mode (-)
ESI location	2,5 mm from the DL line opening
ESI interface voltage	-3,5 kV
Nebulizing Gas	1,1 l/min
Drying Gas	13 l/min
Heat Block Temperature	350 °C
DL Temperature	250 °C

8.3 Calibration

The calibration was done using seven calibration levels and the method in use was fixed calibration. In this method the calibration was performed minimum on three different days during two weeks' time and these different calibrations were combined into one. The accuracy of calibration is considered acceptable if it is between 85–115 per cent for the quantitative analytes. There is no accuracy limit for the semi-quantitative analytes. This process was done once a year or sooner if needed. The validity of the calibration was monitored analysing a reference sample with every sample sequence. (Nousiainen 2015, 18–19.)

The calibration curve used quadratic formula that is not forced to zero. This is since the software used by the analyser only allows one type of formula to be used in one calibration and the semi-quantitative analytes cannot be fitted to a linear formula. This formula uses equation 6.

$$\frac{Area_A}{Area_{ISTD}} = a\left(\frac{[A]}{[ISTD]}\right)^2 + b\frac{[A]}{[ISTD]} + c \quad (6)$$

where

$Area_A$ = the area of the spike of the analyte
$Area_{ISTD}$ = area of the spike of the internal standard
[A] = the concentration of the analyte (mg/l)
[ISTD] = the concentration of the internal standard (mg/l)
a = a response factor
b = a response factor
c = a constant.

The only exceptions to this formula were HMF and furfural which were analysed using the DAD-detector and the constants in regression in the formula were forced to zero. This way they could use linear plot and equation 2. The analyte concentrations from samples were calculated using equation 7. (Nousiainen 2015, 18–19.)

$$[A_{sample}] = \frac{[A]}{m_{sample}} \quad (7)$$

where

$[A_{sample}]$ = sample concentration (mg/kg)
[A] = value calculated using equation 6 (µg)
m_{sample} = the mass of the sample (g).

This calibration was done beforehand and not as a part of this thesis.

9 Execution

The measurements were done in two phases. First one was to choose what sample to use for the second phase where the sample was then analysed as spiked and non-spiked. These phases are explained in chapters 4.2 and 4.3. The sample preparation was done using the instructions in the method procedure with small exceptions with spiked samples.

9.1 Sample Preparation

The sample preparation was done using the liquid-liquid extraction method where first the sample was dissolved in chloroform and then target compounds were extracted from it using water. Full procedure was

1. The sample was heated at 60 °C maximum of 60 minutes.
2. The sample was weighed into a 2 ml Eppendorf tube. Normal amount was 20–400 mg depending on the expected amount of acids in the sample. The exact weight was marked for calculating the results.
3. 60 µl of ISTD was added gravimetrically after the sample had cooled down.
4. 800 µl of chloroform was added to the sample.
5. The sample was mixed in Vortex mixer (speed 7–8) until it had dissolved into the solvent.
6. The sample was centrifuged using fast spin for 10-15 seconds.
7. 350 µl of water was added to the sample.
8. The sample was mixed in Vortex mixer (speed 7–8) for 30 seconds.
9. The sample was centrifuged for 5 minutes at 12000 rpm speed.

10. The top layer which was the water phase was pipetted carefully and filtered through syringe and filter and into a glass vial.

9.2 Analysed Analytes

There were total of 23 short chain carboxylic acids, furfural, and HMF that were analysed from the sample matrix. From these 23 acids, eight compounds could be quantified using internal standards labelled with deuterium. The rest fifteen acids could not be accurately quantified because they relied on the same standards. As different compounds eluate differently from the HPLC column the standards could not completely accommodate the other analytes. This made these analyses semi-quantitative. Both furfural and HMF could be quantified from water-based sample matrix but from oil based samples it could not be quantified at all. The validation parameters were calculated for these two compounds as well as for the short chain carboxylic acids but for accurate results a different method should be used. (Nousiainen 2015,6.)

The eight short chain carboxylic acids that was quantified with this method were

- acetic acid
- propionic acid
- butyric acid
- pentanoic acid
- hexanoic acid
- sodium lactate
- succinic acid
- levulinic acid.

The fifteen other acids that was measured but that could not be quantified accurately were

- formic acid
- glyoxylic acid
- glycolic acid

- pyruvic acid
- isobutyric acid
- isovaleric acid
- maleic acid
- malic acid
- glutaric acid
- galacturonic acid
- a-ketoglutaric acid
- glucuronic acid
- malonic acid
- fumaric acid
- itaconic acid.

9.3 Sample Matrix

Neste has been a forerunner for processing usable oil and fuels from waste material. As more companies have started to do the same, Neste has expanded the materials to include more challenging and impure sources. There are more of this material available because the difficulty and duration of the purification process makes it less desirable material. (Pesonen 2021.)

The sample matrix validated in this study was one of those more challenging materials and as it has no official name, this study will use the term brown grease that is commonly used in Neste. These types of samples come from wastewater in different animal processing facilities and includes food waste such as cheese and cooking fats. (Jin 2021b.)

9.4 First Phase

The first phase of the validation was to analyse four samples selected beforehand by the supervising researcher. The goal was to find a sample that would have low quantities of the compounds so adding spiking solution would not exceed the calibration limits of the analyser. There had to be several preparations of every

sample to find the optimal weight to use. The weight could be anything between 20 to 400 mg depending on the sample. The results of this first phase can be seen in appendix 2. The sample was chosen to be Sample 3, Food Waste Oil.

9.5 Second Phase

In the second phase, the chosen sample was analysed as itself as well as with spiking. These levels are shown in table 3.

Table 3. The sample levels and added amounts of analyte working solution.

Sample Level	Analyte Working Solution (µl)	LC-MS grade water (µl)
0 (non-spiked)	0	350
1	100	250
2	300	50
3	350 (second solution)	0

The first three levels of samples were prepared and analysed three times a day on three different days to count in any possible effects of sample preparation and daily fluctuation. The spiking was done with analyte working solution which was prepared specially for these analyses.

Precision, limit of detection, limit of quantification and trueness were calculated from the results gained from these analyses. After the second phase some of the analytes still produced low accuracies and precisions so for them another analyte working solution was made. The volumes of the stock solutions used in the second analyte working solution were calculated by the supervising researcher. This third spiking was done three different times per day on two different days.

9.6 Analyte Working Solutions

The spiked analyte working solutions were prepared according to the work procedure of the method (Nousiainen 2015, 14–15). There was a crude product of every analyte with 100 per cent purity and they were weighed and dissolved to either water or solution including 1:1 ratio of water and acetonitrile. Some of these analyte stock solutions were made into 5 ml volumetric flasks but most was measured pipetting gravimetrically 4 ml of the solvent to a vial.

The first analyte working solution was made by pipetting these stock solutions gravimetrically into a 5 ml volumetric flask and filling that with an eluent used in the analysis method. The analyte working solution was prepared every day to make sure no evaporation or any other loss would happen while storing the diluted solution.

The second analyte working solution was done to calculate the precision for some analytes. Because some analytes had already produced good accuracies with spiking level one or two, the stock solutions of these analytes were not added to the working solution. The analytes used in this second analyte working solution were butyric acid, isobutyric acid, hexanoic acid, succinic acid, glycolic acid, malonic acid, maleic acid, malic acid, itaconic acid, ketoglutaric acid, glucuronic acid, and furfural. All information about both analyte working solutions and the stock solutions including weighings, used solvents, and total volumes can be seen in appendix 1.

10 Results

The validation samples were analysed three times a day on three different days except the spiking level three which was done separately three times a day on two different days. All the data was collected before any validation parameters were calculated. The results are shown in Appendix 3.

Before calculating any validation parameters, the data was analysed to find any outliers. This was done using Grubbs's test. Grubbs's test can be used for data as long it follows normal distribution. The data is calculated using equation 8. The result is then compared to a critical value that can be found ready in many statistical mathematics workbooks. This critical value depends on how many observations the data includes. If calculated result is higher than the critical value, it is considered to differ from the others so much that it should be removed from the data. The test was done to every level and ever analyte separately using the confidence level of 95 per cent. The calculated and critical values are shown in appendix 4. (Koriseva 2021.)

$$G_{calculated} = \frac{|\bar{Y} - Y_{out}|}{s} \quad (8)$$

where

$\overline{Y}$ = mean
Y_{out} = the value of the data that differs from others the most
s = standard deviation.

This Grubbs's test showed some isolated results to be outliers and those were removed from the data before calculating validation parameters.

10.1 Precision

Precision was calculated for every level using equation 4 and as it is expressed as percentage of relative standard deviation, the smaller the deviation is the better the results are. These are shown in table 4.

Table 4. Precision results.

	Level 0		Level 1		Level 2		Level 3	
Compound	Average Conc. (mg/kg)	RSD (%)	Average Conc. (mg/kg)	RSD (%)	Average Conc. (mg/kg)	RSD (%)	Average Conc. (mg/kg)	RSD (%)
formic acid	9,54	21,1	18,63	11,4	34,24	5,60		
acetic acid	14,62	3,9	19,51	3,5	29,53	3,79		
propanoic acid	2,99	7,9	4,57	6,1	7,56	6,53		
butyric acid	1,69	10,4	3,09	6,8	5,78	7,34	608,53	2,51
isobutyric acid	0,58	11,3	1,91	6,6	4,45	7,87	787,81	4,07
valeric acid	3,13	17,9	4,64	13,7	7,55	11,91		
isovaleric acid	0,37	13,1	2,60	10,7	6,60	9,95		
hexanoic acid	21,20	9,9	23,42	11,7	26,71	11,08	412,42	5,90
lactic acid	0,11	43,7	0,90	8,9	2,36	2,08		
succinic acid	1,71	4,9	1,90	3,9	2,25	4,02	79,43	2,60
levulinic acid	12,30	3,6	13,78	4,0	16,42	2,41		
glyoxylic acid	0,00	-	2,36	8,5	7,01	6,15	316,39	3,28
glycolic acid	0,41	10,5	3,24	8,8	8,48	6,75		
pyruvic acid	0,12	16,1	0,88	4,3	2,45	4,98		
malonic acid	0,00	-	1,77	3,7	2,29	5,59	74,55	2,58
maleic acid	0,03	15,3	0,12	7,5	0,28	9,15	7,32	1,83
fumaric acid	0,00	-	0,34	5,5	0,88	2,58		
malic acid	0,07	7,1	0,09	0,0	0,11	8,66	9,45	8,73
itaconic acid	0,02	21,4	0,03	20,6	0,03	13,89	40,41	5,78
glutaric acid	4,13	8,9	4,28	8,6	4,44	9,82		
ketoglutaric acid	0,07	9,8	0,11	7,7	0,30	12,13	8,33	10,71
glucuronic acid	0,06	13,3	0,07	10,3	0,11	6,63	8,99	3,40
galacturonic acid	0,04	20,1	0,05	57,4	0,09	27,84		
furfural	0,05	5,3	0,07	15,4	0,23	20,38	72,11	3,24
HMF	0,12	19,1	1,53	13,9	3,93	14,50		

Mostly the results follow expected pattern where the precision gets smaller the higher the concentrations are. This is because in low concentrations even small fluctuation causes higher percentage. This means that a plot where y axis shows the RSD and x axis shows the concentration follows a descending trend.

Most of the compounds follow this trend but there were some slight exceptions. These however can be explained on the fact that even in the spiked samples the concentrations were low in some. Others that had concentrations over 10 mg/kg in the zero sample would most likely follow the trend, but the higher RSDs cannot be seen with these starting concentrations. An example of these plots is shown in figure 7 and all can be seen in appendix 5.

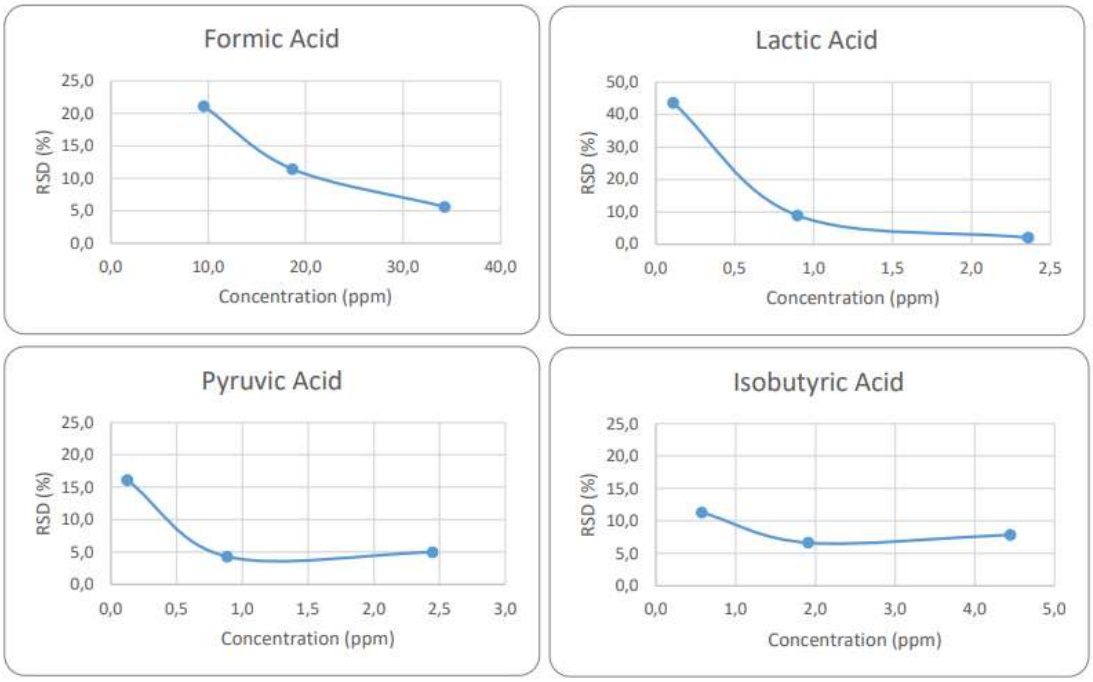

Figure 6. RSD plots for formic acid, lactic acid, pyruvic acid, and isobutyric acid.

Acceptance limit for precision was set on RSD 25 % meaning results above it were rejected. All except galacturonic acid reached this limit on some levels and even this is only due to the small concentrations on the zero sample as well as on all the spiking levels.

10.2 Trueness

Trueness was calculated using equation 5 first from the two different levels of spiked samples. Same as precision, trueness is expressed as percentage and can fluctuate a lot if concentrations are low. As trueness is the relation between the spiked amount and the measured concentration (recovery–%), the best result would be 100 per cent but results are acceptable between 80 and 120 per cent. The comprised results are shown in table 5 and can be seen more widely in appendix 6.

Table 5. Recovery–% results.

	Level 1 (%)	Level 2 (%)	Level 3 (%)
formic acid	97,1	87,9	
acetic acid	89,5	90,9	
propanoic acid	89,7	86,2	
butyric acid	80,5	78,1	99,5
isobutyric acid	51,8	63,9	93,3
valeric acid	81,6	79,8	
isovaleric acid	110,3	102,8	
hexanoic acid	207,8	172,3	118,0
lactic acid	90,8	86,5	
succinic acid	169,0	158,1	129,3
levulinic acid	97,3	90,1	
glyoxylic acid	131,6	130,7	35,3
glycolic acid	117,2	111,2	
pyruvic acid	101,0	102,9	
malonic acid	674,0	291,2	90,1
maleic acid	387,4	360,6	18,0
fumaric acid	131,7	115,6	
malic acid	79,4	64,3	31,6
itaconic acid	2,2	3,3	141,5
glutaric acid	131,4	92,0	
ketoglutaric acid	141,4	311,6	28,8
glucuronic acid	43,7	73,7	4,0
galacturonic acid	80,1	93,5	
furfural	2,2	5,2	6,1
HMF	122,0	110,0	

The table shows relatively good results to some of the compounds but as the concentrations are extremely low on some, the trueness results tend to variate as well. It also needs to be stated that most of the compounds measured are not truly quantifiable and it may have effect on these results too. The only quantitative analyte that did not yield good enough trueness is succinic acid. The reason for this is not clear but it is suspected that it is because the chromatograph's spike for succinic acid is not symmetrical and depending on the integration of the spike, results can vary.

The spiked samples can be also presented in plot form where y axis shows the spiked amount and x axis the measured concentration. From these plots, linearity can be determined. Optimally the different levels of spiked samples should form a linear trend. As there are 25 compounds, only few of these are shown in figure 8 for example. All the plots can be seen in appendix 7.

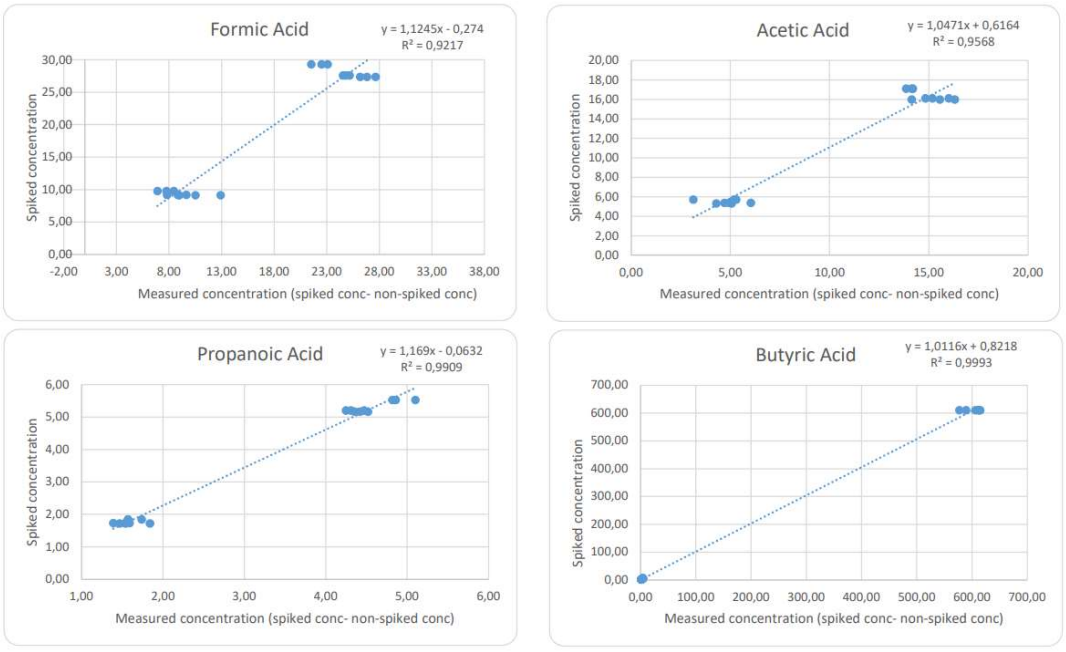

Figure 7. Linearity examples of spiked samples showing plots for formic acid, acetic acid, propanoic acid, and butyric acid.

10.3 Limit of Detection And Limit of Quantification

Limit of detection was determined by first making a plot with the concentrations and their signal-to-noise-ratios and then calculating the formula for the equation. Using this formula, the concentration of any S/N-ratio could be then calculated. Because the S/N-ratio can vary depending on the integration of the spike, all sample results were used. This included the samples and replicates from the first phase of the validation and all the replicates from the second phase. Using more observations, evened the fluctuation. As mentioned in chapters 2.8.3 and 2.8.4, the LOD was the concentration that had the S/N-ratio of 3 and LOQ was the concentration that had the S/N-ratio of 10. All the plots and their formulas can be seen in appendix 8 and the limit values are shown in table 6.

Table 6. LOD and LOQ results.

Compound	LOD (mg/kg)	LOQ (mg/kg)
Formic acid	2,78	2,83
Acetic acid	0,77	2,18
Propanoic acid	1,46	1,54
Butyric acid	2,30	2,46
Isobutyric acid	2,41	2,50
Valeric acid	1,30	2,62
Isovaleric acid	3,76	4,22
Hexanoic acid	2,64	2,91
Lactic acid	0,22	0,23
Succinic acid	1,62	1,64
Levulinic acid	3,22	3,34
Glyoxylic acid	0,09	0,16
Glycolic acid	0,08	0,12
Pyruvic acid	0,11	0,16
Malonic acid	1,75	1,91
Maleic acid	0,08	0,08
Fumaric acid	0,57	0,57
Malic acid	0,08	0,08
Itaconic acid	0,02	0,02
Glutaric acid	3,71	3,71
Ketoglutaric acid	0,05	0,06
Glucuronic acid	0,03	0,04
Galacturonic acid	0,01	0,01
Furfural	0,01	0,05
HMF	0,13	0,15

The LOD and LOQ were determined from this data but even though some analytes were quantified in extremely low concentrations, the LOQ was set on 1 ppm. This was because concentrations lower than that can be hard to calibrate and as they do not influence handling or production of the material, this limit was previously set on 1 ppm for all analytes and sample matrixes.

11 Conclusions

The purpose of this thesis was to validate an existing analysing method for short chain carboxylic acids to a new bio oil based sample matrix in Research Analytics

Team at the Chemistry Department of Neste Oil Research and Development. The method used electrospray ionization and ion exclusion chromatography combined with mass spectrometer as the detector. The validation parameters calculated were precision, trueness, limit of detection, and limit of quantification.

Only eight of the 25 compounds analysed were quantifiable and the rest were either semi-quantitative or nonquantifiable. This means that only those eight quantitative compounds could be expected to yield accurate results, and this was clearly seen as trueness were calculated. Further analyses could benefit the trueness of succinic acid, but the other quantitative analytes were within the limits of 80–120 per cent given to trueness results. If more analyses are not performed succinic acid concentrations can be given with rounding the results, for example to closest tenth. As for the analytes that were not quantitative it is not necessary to get the results within the limits. For this reason, more analyses would not profit these even though the trueness results for some did vary greatly. All the other validation parameters did produce good results and for this the validation project can be said to be successful. Some analytes had very low quantification limits but as the Neste already had set the limit for other sample matrixes to 1 ppm, it should be set the same to this matrix as well to avoid confusion. When it comes to furfural and HMF these cannot be reliably analysed from oil-based samples even though they were included in these calculations. Sample can be said to contain those compounds but for accurate analysis another analysing method should be recommended.

This thesis work gave a great experience and understanding on validation and LC-MS both in theory and in practice. Also, as starting the work was delayed for almost two weeks due to issues with water filtration it did make appreciate how crucial properly cleaned water is to these analyses.

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Analyte Stock Solution And Working Solutions

Stock solutions									Working solution						
Name	Combound	Purity (%)	MW (g/mol)	MW in used material	m (mg)	solvent	V (ml)	mg/ml	V (ml)	m(mg)	ppm	dilution (x80)	Day 1	Day 2	Day 3
Muurahaishappo (C1:0)	formic acid	100	46,00	46,00	252,1	water	5	50,42	0,25	257,27	2594,31	32,43	31,92	32,19	34,18
Etikkahappo (C2:0)	acetic acid	100	60,00	60,00	149,1	water	5	29,82	0,25	254,01	1514,92	18,94	18,64	18,79	19,96
Propaanihappo (Propaanoic, C3:0)	propanpoic acid	100	74,00	74,00	16,24	water	4	4,06	0,60	602,69	489,38	6,12	6,02	6,07	6,45
Butaanihappo (Butyric, C4:0)	butyric acid	100	88,00	88,00	48,37	water	4	12,09	0,20	200,37	484,59	6,06	5,96	6,01	6,38
2-Metyylipropaanihappo (Isobutyric, C4:0)	isobutyric acid	100	88,00	88,00	47,58	water	4	11,90	0,20	300,38	714,60	8,93	8,79	8,87	9,41
Pentaanihappo (Valeric, C5:0)	valeric acid	100	102,00	102,00	16,95	water	4	4,24	0,60	603,45	511,42	6,39	6,29	6,35	6,74
3-Metyylibutaanihappo (Isovaleric, C5:0)	isovaleric acid	100	102,00	102,00	18,55	water	4	4,64	0,60	603,59	559,83	7,00	6,89	6,95	7,38
Heksaanihappo (C6:0)	hexanoic acid	100	116,00	116,00	19,81	water:ACN 50:50	4	4,95	0,30	269,99	295,13	3,69	3,63	3,66	3,89
2-Hydroksipropaanihappo (Lactic, C3:0-OH)	lactic acid	100	90,00	112,06	17,06	water	4	3,43	0,35	350,72	240,27	3,00	2,96	2,98	3,17
Butaanidihappo (Succinic, C4:0-di)	succinic acid	100	118,00	118,00	16,07	water	4	4,02	0,04	40,05	32,18	0,40	0,40	0,40	0,42
Levuliinihappo (C5:0-KETO)	levulinic acid	100	116,12	116,12	105,31	water	5	21,06	0,10	100,17	421,96	5,27	5,19	5,24	5,56
Oksoetaanihappo (Glyoxylic, C2:0-ALDO)	glyoxylic acid	100	74,00	92,05	48,3	water	4	9,71	0,25	255,43	495,90	6,20	6,10	6,15	6,53
Hydroksietaanihappo (Glycolic, C2:0-OH)	glycolic acid	100	76,00	76,00	52,59	water	4	13,15	0,25	254,97	670,44	8,38	8,25	8,32	8,83
2-Oksopropaanihappo (Pyruvic, C3:0-KETO)	pyruvic acid	100	88,00	110,04	14,81	water	4	2,96	0,35	351,96	208,43	2,61	2,56	2,59	2,75
Propaanidihappo (Malonic, C3:0-di)	malonic acid	100	104,06	104,06	14,6	water	4	3,65	0,10	99,34	72,52	0,91	0,89	0,90	0,96
cis-Buteenidihappo (Maleic, C4:1-di)	maleic acid	100	116,00	116,00	13,3	water	4	3,33	0,01	9,70	6,45	0,08	0,08	0,08	0,08
Trans-Buteenidihappo (Fumaric, C4:1-di)	fumaric acid	100	116,00	116,00	15,01	water:ACN 50:50	4	3,75	0,10	85,03	70,42	0,88	0,87	0,87	0,93
Hydroksibutaanidihappo (Malic, C4:0-OH-di)	malic acid	100	134,00	134,00	11,65	water	4	2,91	0,01	9,43	5,49	0,07	0,07	0,07	0,07
Metyleenibutaanidihappo (Itaconic, C5:1-di)	itaconic acid	100	130,00	130,00	14,17	water	4	3,54	0,04	40,02	28,35	0,35	0,35	0,35	0,37
Pentaanidihappo (Glutaric, C5:0-di)	glutaric acid	100	132,00	132,00	14,78	water	4	3,70	0,04	41,29	30,51	0,38	0,38	0,38	0,40
2-Oksopentaanidihappo (Ketoglutaric, C5:0-KETO-di)	ketoglutaric acid	100	146,00	146,00	14,01	water	4	3,50	0,01	9,58	6,71	0,08	0,08	0,08	0,09
Glukuronihappo (C6-uroni)	glucuronic acid	100	194,00	194,00	12,67	water	4	3,17	0,01	9,39	5,95	0,07	0,07	0,07	0,08
Galakturonihappo (C6-uroni)	galacturonic acid	100	194,00	216,12	13,12	water	4	2,94	0,01	9,20	5,42	0,07	0,07	0,07	0,07
Furfural	furfural	100	96,08	96,08	97,72	water	5	19,54	0,80	80,22	313,56	3,92	3,86	3,89	4,13
Hydroxymethylfurfural	HMF	100	126,11	126,11	100,78	water	5	20,16	0,80	79,35	319,88	4,00	3,94	3,97	4,21

Water:ACN 50:50	
V (ml)	4
m (g)	3,624567
density (g/cm³)	0,906142

Working solution	
V (ml)	5

~~Analyte Working Solution 2:0~~				Analyte Working Solution 2:1		
~~V (ml)~~	~~m (mg)~~	~~ppm~~	V (ml)	m (mg)	ppm
~~0~~	~~0~~	~~0~~	0	0	0
~~0~~	~~0~~	~~0~~	0	0	0
~~0~~	~~0~~	~~0~~	0	0	0
~~0,40~~	~~399,43~~	~~966,02~~	0,25	252,30	610,19
~~0,60~~	~~596,18~~	~~1415,93~~	0,35	354,65	843,71
~~0~~	~~0~~	~~0~~	0	0	0
~~0~~	~~0~~	~~0~~	0	0	0
~~0,60~~	~~543,06~~	~~536,91~~	0,35	309,37	338,17
~~0~~	~~0~~	~~0~~	0	0	0
~~0,70~~	~~694,87~~	~~550,33~~	0,07	72,62	58,35
~~0~~	~~0~~	~~0~~	0	0	0
~~0,45~~	~~453,51~~	~~880,46~~	0,45	457,12	887,47
~~0~~	~~0~~	~~0~~	0	0	0
~~0~~	~~0~~	~~0~~	0	0	0
~~0,35~~	~~348,46~~	~~354,38~~	0,11	112,41	82,06
~~0,45~~	~~449,99~~	~~299,18~~	0,06	60,77	40,41
~~0~~	~~0~~	~~0~~	0	0	0
~~0,25~~	~~248,72~~	~~344,88~~	0,05	50,93	29,67
~~0,35~~	~~348,39~~	~~247,42~~	0,04	40,89	28,97
~~0~~	~~0~~	~~0~~	0	0	0
~~0,10~~	~~98,45~~	~~68,96~~	0,04	40,94	28,68
~~0,45~~	~~449,77~~	~~284,93~~	0,35	351,46	222,65
~~0~~	~~0~~	~~0~~	0	0	0
~~0,30~~	~~303,36~~	~~1181,86~~	0,30	303,26	1185,38
~~0~~	~~0~~	~~0~~	0	0	0

Results from The First Phase of Validation

Sample weight (mg) →	Sample 1 - 15400747				Sample 2 - 15418005					Sample 3 - 15416981					Sample 4 - 1541682				
	50	75	100	Average	20	100	200	400	Average	100	400	100	400	Average	20	100	200	400	Average
Analyte ↓																			
Formic acid	2,97	11,34	16,36	**11,34**	-15,52	10,67	10,98	15,91	**12,52**	-1,03	5,77	-0,6	5,49	**5,63**	25,58	53,43	55,03	57,9	**47,99**
Acetic acid	40,38	58,28	59,95	**58,28**	0,00	0,00	5,76	5,86	**5,81**	0	13,44	0	13,42	**13,43**	0	0	18,64	24,7	**24,70**
Propionic acid	374,44	369,05	384,25	**379,35**	20,36	24,77	24,15	24,61	**23,47**	1,91	2,52	2,18	2,43	**2,26**	2,36	4,23	4	4,76	**4,33**
Butyric acid	246,79	257,33	256,94	**251,87**	5,72	7,28	6,75	7,32	**6,77**	1,31	1,27	1,09	1,36	**1,26**	2,34	3,5	3,25	3,66	**3,19**
Isobutyric acid	6,05	4,57	6,34	**5,65**	0,73	0,79	0,94	0,72	**0,80**	0,41	0,32	0,4	0,35	**0,37**	1,09	0,81	1,05	1,1	**0,99**
Valeric acid	429,02	441,73	462,26	**445,64**	77,35	80,45	83,89	82,58	**81,07**	0	2,23	0	2,26	**2,25**	0	0	3,04	3,07	**3,06**
Isovaleric acid	30,72	31,24	32,74	**31,73**	5,00	5,03	5,54	5,20	**5,19**	0	0	0	0	**0,00**	0	0	4,29	3,87	**4,08**
Hexanoic acid	111,44	109,14	107,07	**109,26**	38,64	45,10	41,99	41,41	**41,79**	21,56	19,14	21,08	18,6	**20,10**	129,38	150,63	148,97	190,98	**154,99**
Lactic acid	37,77	42,54	39,23	**38,50**	1,00	1,36	0,88	1,22	**1,12**	0,13	0,18	-0,04	0,01	**0,16**	1,94	1,54	1,56	1,52	**1,64**
Succinic acid	4,66	5,81	5,58	**5,35**	1,32	1,30	0,88	0,72	**0,80**	1,7	1,53	1,71	1,56	**1,63**	1,85	1,57	1,3	1,45	**1,54**
Levulinic acid	2,27	1,45	1,31	**1,68**	2,68	1,14	0,54	0,40	**1,19**	14,61	14,21	14,49	13,98	**14,32**	5,99	4,46	3,85	4,29	**4,65**
Glyoxylic acid	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**
Glycolic acid	0	0	0	**0**	0	0	0,47	0,43	**0,45**	0	0,29	0	0,26	**0,28**	0	1,57	1,45	1,37	**1,46**
Pyruvic acid	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0,16	0,1	0,07	**0,11**
Malonic acid	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**
Maleic acid	0,11	0,12	0,11	**0,11**	0	0	0,01	0,03	**0,02**	0,01	0,01	0	0,01	**0,01**	0	0	0	0	**0**
Fumaric acid	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**
Malic acid	0	0	0	**0**	0	0	0,14	0,08	**0,11**	0	0	0	0	**0**	0	0	0	0,08	**0,08**
Itaconic acid	11,85	4,21	7,40	**7,82**	0,02	0,04	0	0	**0,02**	0	0	0	0,07	**0,07**	0,08	0,05	0,01	0,11	**0,11**
Glutaric acid	0	0	1,12	**1,12**	0	0,37	0,37	0,37	**0,37**	3,57	3,77	3,68	4,08	**3,78**	2,59	2,17	2,27	2,33	**2,26**
Ketoglutaric acid	0	0	0	**0**	0	0	0,05	0,04	**0,02**	0	0,03	0	0,02	**0,03**	0	0	0	0,02	**0,02**
Glucuronic acid	0,14	0,19	0,14	**0,16**	0	0	0,02	0	**0,01**	0	0	0,03	0,01	**0,02**	0	0	0,02	0,02	**0,02**
Galacturonic acid	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**

Results under calibration limit are marked as red
These are not included in the averages unless all results are under limit.

Results Before Data Analysis

	Level 0									Level 1								
Combound	Day 1 - 1	Day 1 - 2	Day 1 - 3	Day 2 -1	Day 2 -2	Day 2 -3	Day 3 - 1	Day 3 - 2	Day 3 - 3	Day 1 - 1	Day 1 - 2	Day 1 - 3	Day 2 -1	Day 2 -2	Day 2 -3	Day 3 - 1	Day 3 - 2	Day 3 - 3
formic acid	9,31	9,05	9,05	7,85	7,95	8,51	8,36	12,07	13,75	18,28	19,56	21,96	17,49	16,78	16,32	16,12	20,53	20,65
acetic acid	15,69	14,97	14,58	13,77	14,62	14,89	14,19	14,79	14,06	19,98	20,03	22,21	19,80	19,32	19,82	19,37	17,92	19,35
propanpoic acid	2,80	2,87	2,84	2,85	2,81	2,90	3,08	3,35	3,41	4,34	4,34	4,68	4,44	4,35	4,29	4,82	4,92	4,98
butyric acid	1,58	1,56	1,60	1,61	1,57	1,58	1,72	1,92	2,04	2,89	2,81	3,24	2,93	3,15	2,90	3,23	3,38	3,31
isobutyric acid	0,54	0,60	0,53	0,51	0,53	0,51	0,68	0,64	0,64	1,85	1,78	2,01	1,79	1,88	1,76	1,99	2,08	2,07
valeric acid	2,68	2,70	2,69	2,93	2,85	2,84	3,45	3,90	4,15	4,12	4,07	4,33	4,26	4,39	4,19	5,19	5,62	5,58
isovaleric acid	0,34	0,35	0,34	0,33	0,33	0,33	0,41	0,43	0,45	2,45	2,29	2,77	2,28	2,44	2,39	2,93	2,91	2,91
hexanoic acid	18,31	20,67	20,85	21,96	21,98	21,49	18,71	21,27	25,60	22,28	22,58	20,88	22,40	22,33	24,01	20,52	28,47	27,29
lactic acid	0,07	0,09	0,11	0,18	0,16	0,16	0,06	0,10	0,05	0,85	0,87	1,05	0,92	0,98	0,92	0,86	0,80	0,82
succinic acid	1,66	1,67	1,69	1,82	1,76	1,76	1,54	1,68	1,77	1,85	1,88	1,95	1,93	2,00	1,95	1,75	1,94	1,86
levulinic acid	12,95	12,32	12,19	12,42	12,58	12,76	11,98	11,48	12,03	13,77	14,32	14,76	14,11	13,72	13,75	13,37	12,93	13,31
glyoxylic acid	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	2,57	2,46	3,23	2,40	2,54	2,37	2,18	2,03	2,13
glycolic acid	0,38	0,38	0,40	0,37	0,38	0,38	0,42	0,50	0,57	3,09	3,07	3,84	2,97	3,12	2,95	3,43	3,32	3,40
pyruvic acid	0,14	0,14	0,12	0,13	0,10	0,10	0,15	0,14	0,10	0,90	0,94	1,24	0,92	0,89	0,84	0,84	0,85	0,86
malonic acid	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	1,71	1,65	1,85	1,84	1,77	1,82	1,73	1,76	1,77
maleic acid	0,04	0,03	0,04	0,04	0,03	0,03	0,03	0,03	0,04	0,12	0,11	0,18	0,13	0,14	0,12	0,12	0,12	0,13
fumaric acid	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,33	0,32	0,46	0,35	0,37	0,34	0,31	0,34	0,33
malic acid	0,07	0,08	0,07	0,08	0,08	0,07	0,07	0,08	0,07	0,09	0,09	0,09	0,09	0,09	0,09	0,09	0,08	0,09
itaconic acid	0,02	0,02	0,02	0,02	0,02	0,02	0,03	0,03	0,03	0,02	0,02	0,02	0,03	0,02	0,03	0,03	0,03	0,03
glutaric acid	3,90	3,84	3,99	4,04	3,82	3,96	4,13	4,70	4,82	4,04	4,05	4,03	3,99	4,15	4,12	4,33	4,98	4,81
ketoglutaric acid	0,08	0,08	0,06	0,08	0,07	0,07	0,07	0,08	0,08	0,11	0,11	0,20	0,11	0,12	0,10	0,12	0,10	0,10
glucuronic acid	0,07	0,06	0,06	0,07	0,05	0,06	0,06	0,05	0,05	0,07	0,08	0,10	0,07	0,07	0,06	0,07	0,06	0,06
galacturonic acid	0,05	0,03	0,04	0,04	0,03	0,04	0,05	0,04	0,03	0,07	0,08	0,10	0,03	0,01	0,01	0,07	0,06	0,06
furfural	0,052	0,046	0,050	0,047	0,045	0,045	0,049	0,051	0,048	0,092	0,051	0,066	0,073	0,069	0,077	0,081	0,070	0,075
HMF	0,151	0,134	0,116	0,108	0,097	0,085	0,117	0,126	0,154	1,951	1,104	1,507	1,505	1,516	1,585	1,527	1,538	1,528

	Level 2								Level 3					
Combound	Day 1 - 1	Day 1 - 2	Day 1 - 3	Day 2 -1	Day 2 -2	Day 2 -3	Day 3 - 1	Day 3 - 2	Day 3 - 3	Day 1 - 1	Day 1 - 2	Day 1 - 3	Day 2 -1	Day 2 -2	Day 2 -3
formic acid	35,49	35,87	36,69	32,65	32,49	33,68	30,87	35,16	35,27	37,03	36,15	34,68	15,90	44,58	45,83
acetic acid	29,83	30,53	30,89	28,6	29,8	30,9	28,05	28,99	28,2	21,13	21,35	21,16	14,05	16,51	18,74
propanpoic acid	7,16	7,39	7,26	7,16	7,28	7,15	8,18	8,17	8,27	4,62	4,71	4,77	3,51	4,49	4,91
butyric acid	5,48	5,4	5,5	5,64	5,57	5,44	6,27	6,29	6,45	613,32	578,69	614,37	590,93	616,22	607,81
isobutyric acid	4,18	4,28	4,28	4,21	4,22	4,12	4,89	4,88	4,95	817,86	798,90	825,30	745,96	781,48	757,35
valeric acid	6,82	6,9	6,89	7,02	7,17	6,99	8,46	8,59	9,11	5,45	5,47	5,43	2,64	6,05	6,06
isovaleric acid	5,97	6,18	6,32	5,91	6,46	6,25	7,71	7,23	7,34	2,05	1,90	1,86	1,18	1,99	2,34
hexanoic acid	24,24	25,72	26,45	26,78	27,12	24,55	22,86	30,82	31,84	458,66	425,16	396,60	377,34	431,01	431,99
lactic acid	2,29	2,29	2,34	2,39	2,4	2,36	2,44	2,35	2,37	0,40	0,40	0,44	0,13	0,14	0,15
succinic acid	2,2	2,23	2,23	2,4	2,27	2,38	2,1	2,24	2,24	78,24	75,39	75,70	74,03	81,44	78,24
levulinic acid	16,37	16,19	17,18	16,25	16,21	16,82	16,32	15,83	16,58	11,77	11,59	11,61	12,46	11,98	12,95
glyoxylic acid	7,6	7,13	7,49	7,23	6,98	7,2	6,57	6,47	6,44	307,04	295,62	303,34	322,97	322,64	325,98
glycolic acid	8,16	8,25	8,31	8,05	7,96	7,91	9,3	9,26	9,09	5,22	5,23	5,14	5,49	5,77	5,94
pyruvic acid	2,49	2,51	2,63	2,34	2,56	2,42	2,49	2,25	2,33	0,10	0,11	0,17	0,21	0,19	0,19
malonic acid	2,18	2,08	2,23	2,22	2,47	2,29	2,43	2,41	2,3	72,89	70,80	72,19	76,74	75,37	75,56
maleic acid	0,27	0,23	0,32	0,3	0,28	0,29	0,29	0,26	0,29	7,43	7,17	7,30	7,46	7,40	7,15
fumaric acid	0,86	0,84	0,91	0,89	0,88	0,87	0,91	0,89	0,89	5,90	6,59	6,68	5,59	6,68	5,02
malic acid	0,12	0,11	0,1	0,1	0,12	0,1	0,12	0,12	0,12	9,15	8,59	8,64	9,47	10,25	10,58
itaconic acid	0,03	0,03	0,03	0,03	0,03	0,03	0,05	0,04	0,04	44,08	42,65	43,24	38,58	39,19	38,41
glutaric acid	4,21	4,06	4,28	4,26	4,08	4,09	4,71	5,04	5,2	8,41	8,41	8,69	4,74	7,33	7,57
ketoglutaric acid	0,31	0,24	0,37	0,29	0,3	0,29	0,34	0,28	0,3	7,67	7,48	7,71	9,72	9,04	8,35
glucuronic acid	0,11	0,11	0,12	0,1	0,1	0,11	0,11	0,1	0,1	8,75	8,44	8,66	9,43	9,11	9,00
galacturonic acid	0,11	0,11	0,12	0,06	0,06	0,06	0,12	0,1	0,1	0,94	0,91	0,96	1,05	1,08	0,99
furfural	0,202	0,143	0,198	0,240	0,286	0,193	0,463	0,257	0,255	74,66	71,48	75,34	69,73	71,16	70,27
HMF	4,130	2,515	3,875	4,021	4,098	4,028	4,309	4,153	5,335	0,32	0,27	0,33	0,13	0,41	0,23

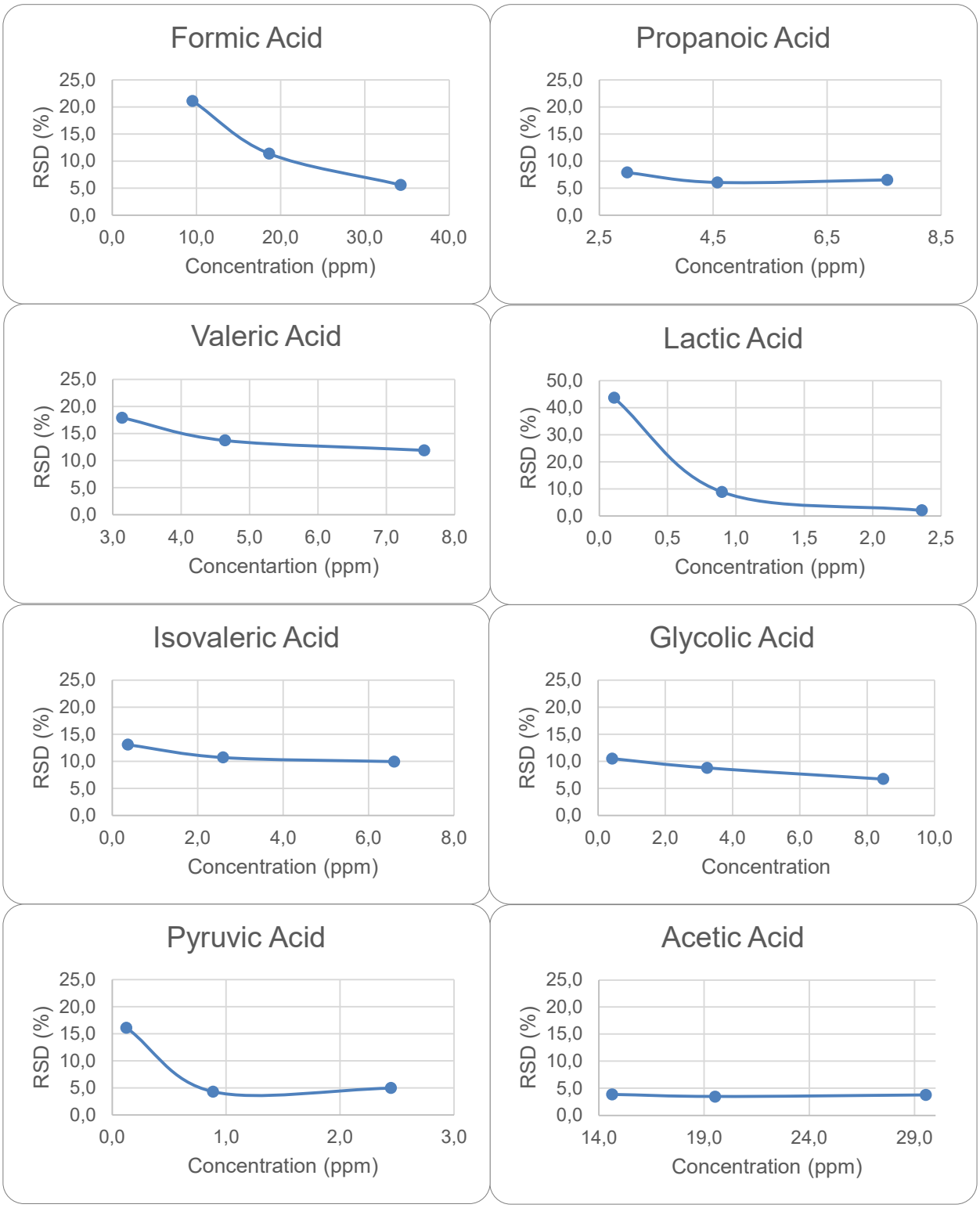
Grubbs's Test Results in Confidence Level 95 Per Cent And Critical Values

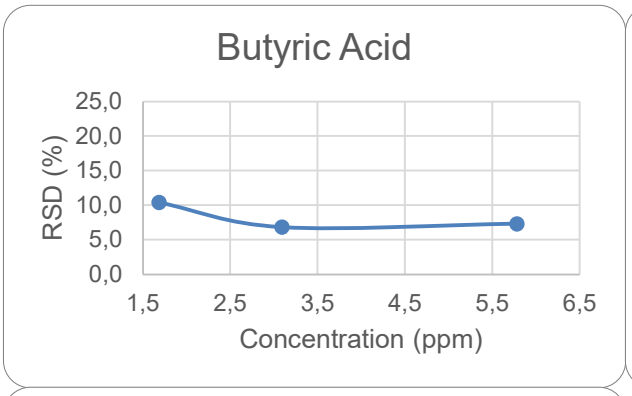
Analyte	Level	G calculated	G critical
formic acid	0	2,09	2,11
	1	1,57	2,11
	2	1,76	2,11
acetic acid	0	1,88	2,11
	1	2,19	2,11
	2	1,33	2,11
propanoic acid	0	1,78	2,11
	1	1,47	2,11
	2	1,44	2,11
butyric acid	0	2,01	2,11
	1	1,35	2,11
	2	1,57	2,11
	3	1,63	1,822
isobutyric acid	0	1,61	2,11
	1	1,32	2,11
	2	1,44	2,11
	3	1,17	1,822
valeric acid	0	1,81	2,11
	1	1,54	2,11
	2	1,73	2,11
isovaleric acid	0	1,71	2,11
	1	1,20	2,11
	2	1,13	2,11
hexanoic acid	0	2,08	2,11
	1	1,85	2,11
	2	1,73	2,11
	3	1,48	1,822
lactic acid	0	1,50	2,11
	1	1,92	2,11
	2	1,40	2,11
succinic acid	0	2,00	2,11
	1	2,03	2,11
	2	1,71	2,11
	3	1,6	1,822
levulinic acid	0	1,84	2,11
	1	1,76	2,11
	2	1,93	2,11
glyoxylic acid	0	n/a	2,11
	1	2,26	2,11
	2	1,33	2,11

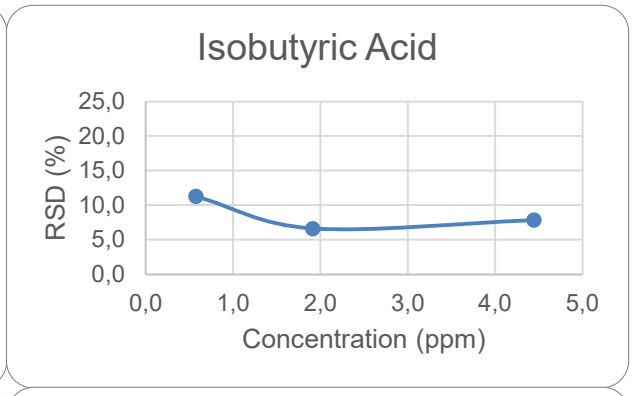
	3	1,38	1,822
glycolic acid	0	2,17	2,11
	1	2,09	2,11
	2	1,44	2,11
pyruvic acid	0	1,27	2,11
	1	2,56	2,11
	2	1,62	2,11
malonic acid	0	n/a	2,11
	1	1,81	2,11
	2	1,64	2,11
	3	1,36	1,822
maleic acid	0	1,05	2,11
	1	2,43	2,11
	2	1,99	2,11
	3	1,26	1,822
fumaric acid	0	n/a	2,11
	1	2,46	2,11
	2	1,85	2,11
malic acid	0	1,05	2,11
	1	2,67	2,11
	2	1,26	2,11
	3	1,37	1,822
itaconic acid	0	1,33	2,11
	1	1,05	2,11
	2	2,14	2,11
	3	1,19	1,822
glutaric acid	0	1,53	2,11
	1	0,79	2,11
	2	1,39	2,11
ketoglutaric acid	0	1,99	2,11
	1	2,58	2,11
	2	1,85	2,11
	3	1,56	1,822
glucuronic acid	0	1,42	2,11
	1	2,28	2,11
	2	1,89	2,11
	3	1,50	1,822
galacturonic acid	0	1,42	2,11
	1	1,46	2,11
	2	1,28	2,11
furfural	0	1,51	2,11
	1	1,94	2,11
	2	2,35	2,11
	3	1,09	1,822
HMF	0	1,55	2,11
	1	1,99	2,11

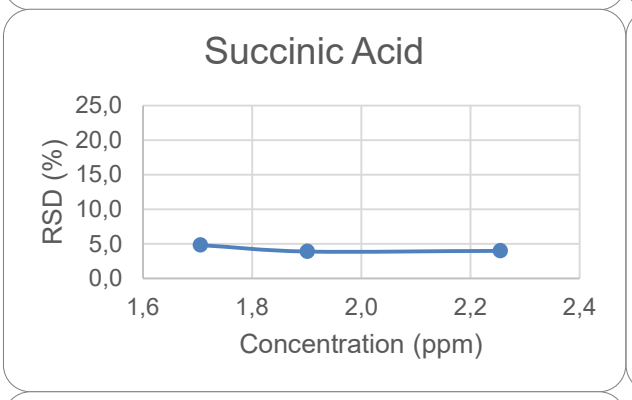
	2	2,14	2,11

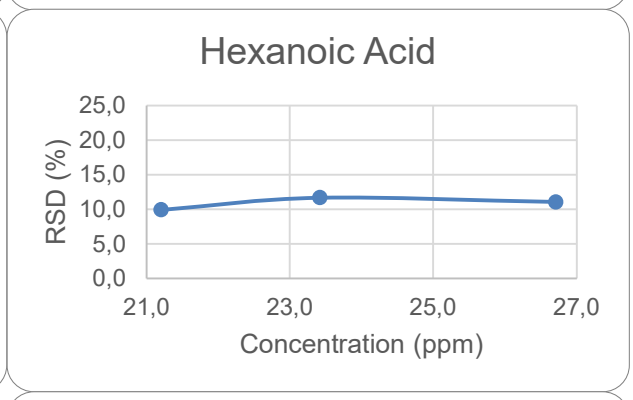
RSD Plots

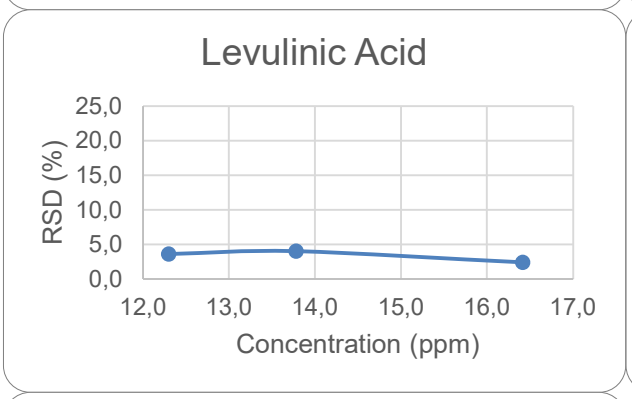

Formic Acid
RSD (%)
25,0
20,0
15,0
10,0
5,0
0,0
0,0
10,0
20,0
30,0
40,0
Concentration (ppm)
Propanoic Acid
RSD (%)
25,0
20,0
15,0
10,0
5,0
0,0
2,5
4,5
6,5
8,5
Concentration (ppm)
Valeric Acid
RSD (%)
25,0
20,0
15,0
10,0
5,0
0,0
3,0
4,0
5,0
6,0
7,0
8,0
Concentartion (ppm)
Lactic Acid
RSD (%)
50,0
40,0
30,0
20,0
10,0
0,0
0,0
0,5
1,0
1,5
2,0
2,5
Concentration (ppm)
Isovaleric Acid
RSD (%)
25,0
20,0
15,0
10,0
5,0
0,0
0,0
2,0
4,0
6,0
8,0
Concentration (ppm)
Glycolic Acid
RSD (%)
25,0
20,0
15,0
10,0
5,0
0,0
0,0
2,0
4,0
6,0
8,0
10,0
Concentration
Pyruvic Acid
RSD (%)
25,0
20,0
15,0
10,0
5,0
0,0
0,0
1,0
2,0
3,0
Concentration (ppm)
Acetic Acid
RSD (%)
25,0
20,0
15,0
10,0
5,0
0,0
14,0
19,0
24,0
29,0
Concentration (ppm)

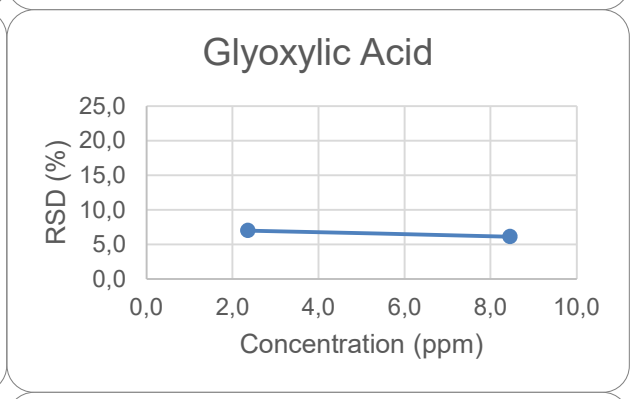

Butyric Acid
RSD (%)
25,0
20,0
15,0
10,0
5,0
0,0
1,5
2,5
3,5
4,5
5,5
6,5
Concentration (ppm)

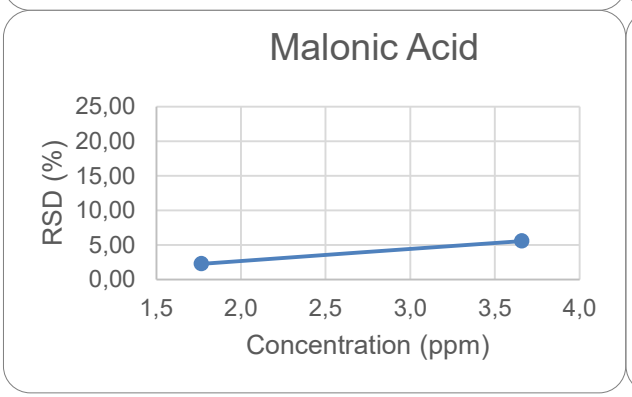

Isobutyric Acid
RSD (%)
25,0
20,0
15,0
10,0
5,0
0,0
0,0
1,0
2,0
3,0
4,0
5,0
Concentration (ppm)

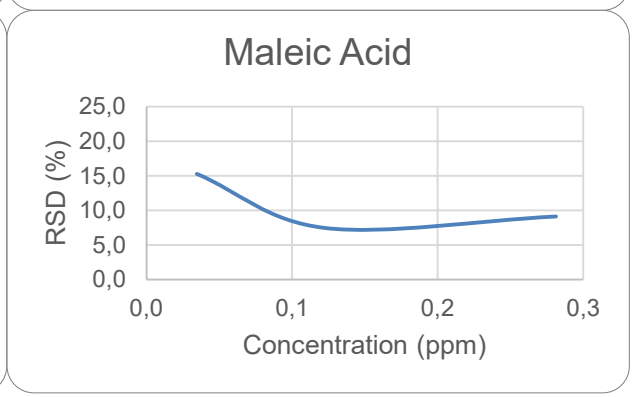

Succinic Acid
RSD (%)
25,0
20,0
15,0
10,0
5,0
0,0
1,6
1,8
2,0
2,2
2,4
Concentration (ppm)

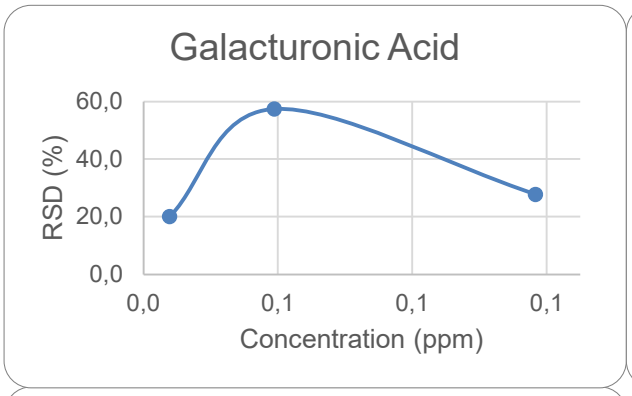

Hexanoic Acid
RSD (%)
25,0
20,0
15,0
10,0
5,0
0,0
21,0
23,0
25,0
27,0
Concentration (ppm)

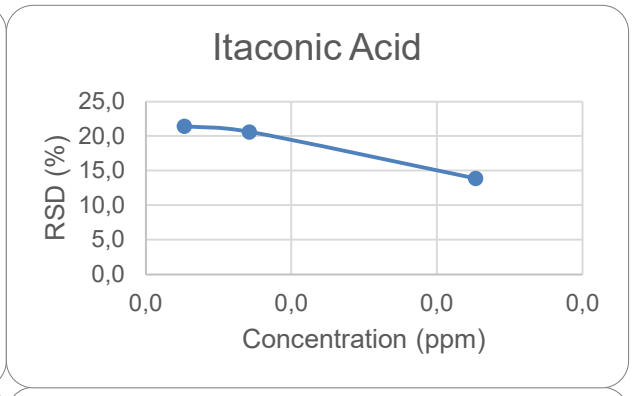

Levulinic Acid
RSD (%)
25,0
20,0
15,0
10,0
5,0
0,0
12,0
13,0
14,0
15,0
16,0
17,0
Concentration (ppm)

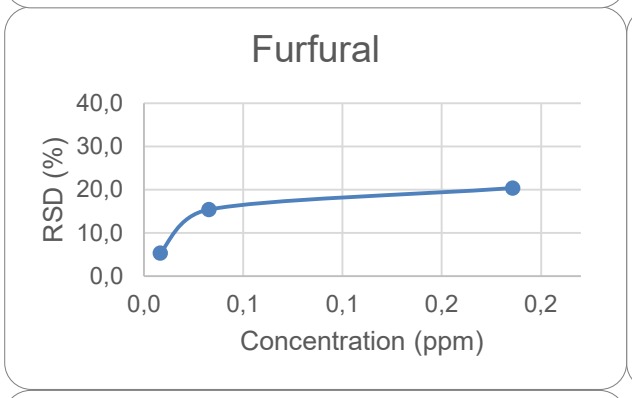

Glyoxylic Acid
RSD (%)
25,0
20,0
15,0
10,0
5,0
0,0
0,0
2,0
4,0
6,0
8,0
10,0
Concentration (ppm)

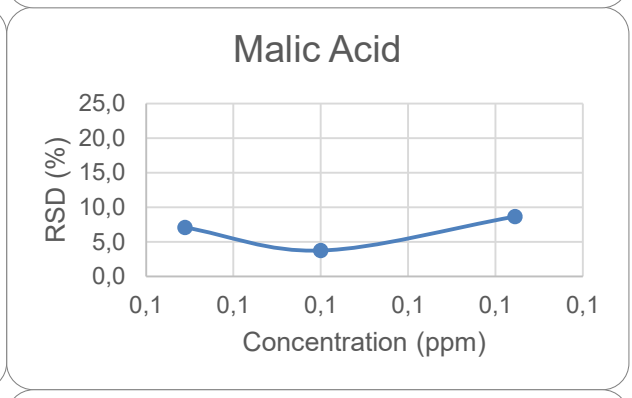

Malonic Acid
RSD (%)
25,00
20,00
15,00
10,00
5,00
0,00
1,5
2,0
2,5
3,0
3,5
4,0
Concentration (ppm)

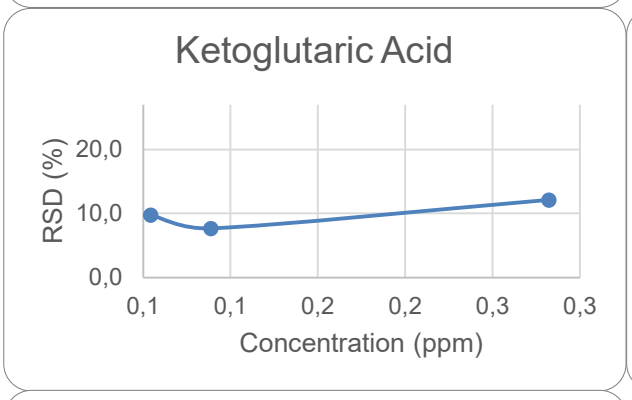

Maleic Acid
RSD (%)
25,0
20,0
15,0
10,0
5,0
0,0
0,0
0,1
0,2
0,3
Concentration (ppm)

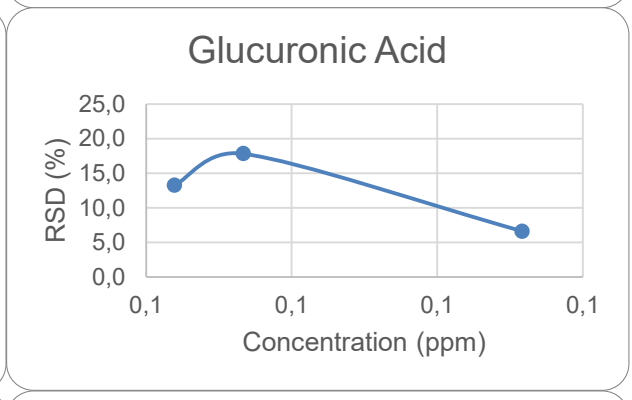

Galacturonic Acid
RSD (%)
60,0
40,0
20,0
0,0
0,0
0,1
0,1
0,1
Concentration (ppm)

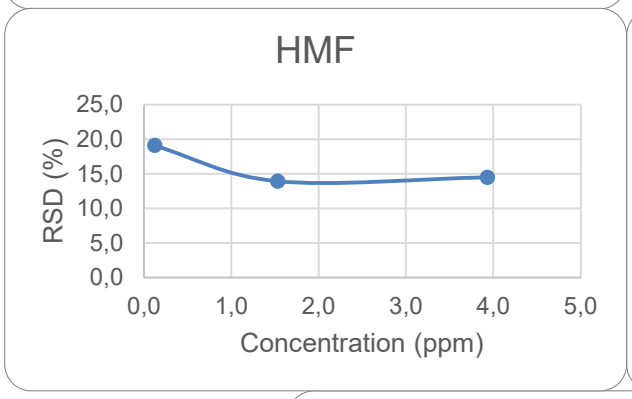

Itaconic Acid
RSD (%)
25,0
20,0
15,0
10,0
5,0
0,0
0,0
0,0
0,0
0,0
Concentration (ppm)

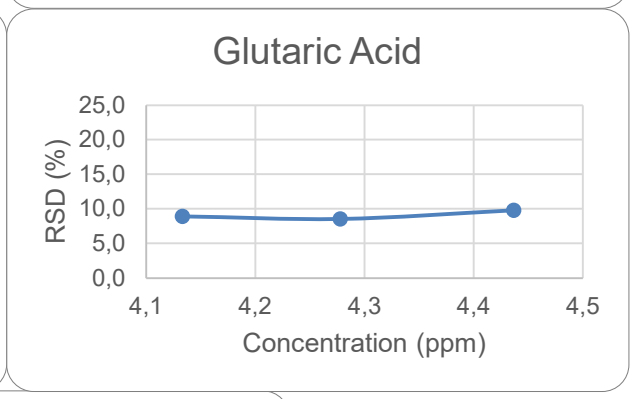

Furfural
RSD (%)
40,0
30,0
20,0
10,0
0,0
0,0
0,1
0,1
0,2
0,2
Concentration (ppm)

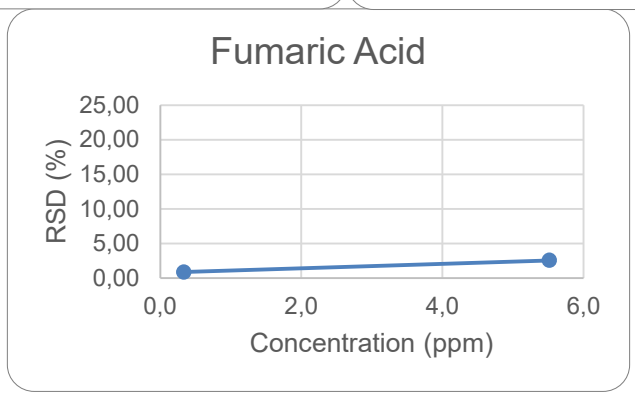

Malic Acid
RSD (%)
25,0
20,0
15,0
10,0
5,0
0,0
0,1
0,1
0,1
0,1
0,1
0,1
Concentration (ppm)


Ketoglutaric Acid
RSD (%)
20,0
10,0
0,0
0,1
0,1
0,2
0,2
0,3
0,3
Concentration (ppm)


Glucuronic Acid
RSD (%)
25,0
20,0
15,0
10,0
5,0
0,0
0,1
0,1
0,1
0,1
Concentration (ppm)


HMF
RSD (%)
25,0
20,0
15,0
10,0
5,0
0,0
0,0
1,0
2,0
3,0
4,0
5,0
Concentration (ppm)

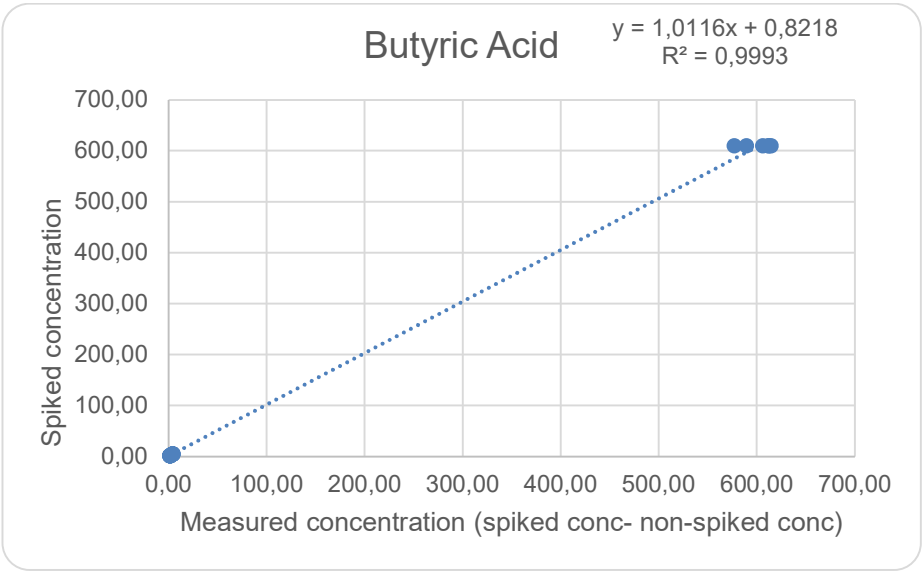

Glutaric Acid
RSD (%)
25,0
20,0
15,0
10,0
5,0
0,0
4,1
4,2
4,3
4,4
4,5
Concentration (ppm)

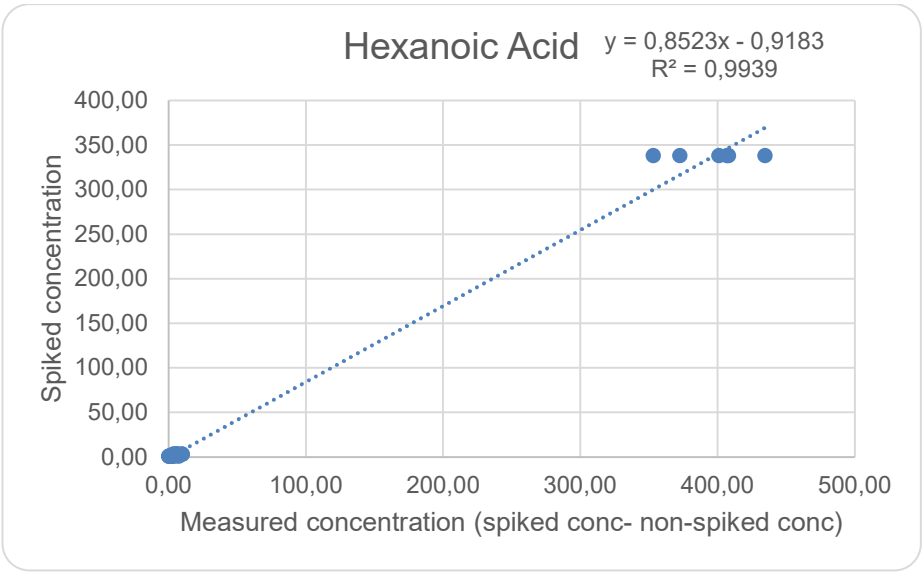

Fumaric Acid
RSD (%)
25,00
20,00
15,00
10,00
5,00
0,00
0,0
2,0
4,0
6,0
Concentration (ppm)

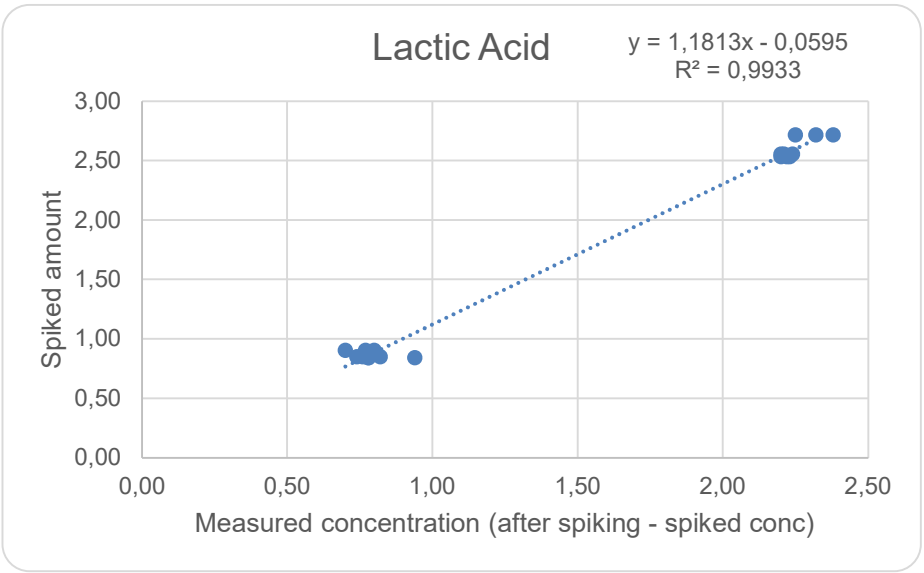
Recovery–% Results

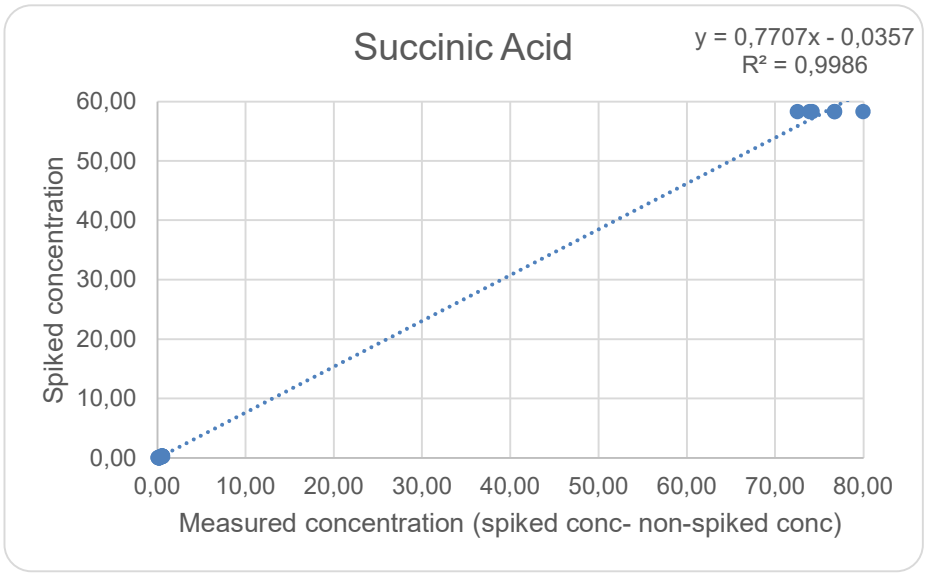
	LEVEL 0	LEVEL 1			LEVEL 2			LEVEL 3		
	Average measured concentration (mg/kg)	Average measured concentration (mg/kg)	Average Spiked Amount - Level 1	Accuracy (%)	Average measured concentration (mg/kg)	Average Spiked Amount - Level 2	Accuracy (%)	Average measured concentration (mg/kg)	Spiked Amount - Level 3	Accuracy (%)
formic acid	9,5	18,63	9,36	97,1	34,24	28,08	87,9			
acetic acid	14,6	19,51	5,47	89,5	29,53	16,40	90,9			
propanoic acid	3,0	4,57	1,77	89,7	7,56	5,30	86,2			
butyric acid	1,7	3,09	1,75	80,5	5,78	5,24	78,1	608,53	610,19	99,45
isobutyric acid	0,6	1,91	2,58	51,8	4,45	6,06	63,9	787,81	843,71	93,31
valeric acid	3,1	4,64	1,85	81,6	7,55	5,54	79,8			
isovaleric acid	0,4	2,60	2,02	110,3	6,60	6,06	102,8			
hexanoic acid	21,2	23,42	1,07	207,8	26,71	3,20	172,3	420,13	338,17	117,96
lactic acid	0,1	0,90	0,87	90,8	2,36	2,60	86,5			
succinic acid	1,7	1,90	0,12	169,0	2,25	0,35	158,1	77,17	58,35	129,34
levulinic acid	12,3	13,78	1,52	97,3	16,42	4,57	90,1			
glyoxylic acid	0,0	2,36	1,79	131,6	7,01	5,37	130,7	312,93	887,47	35,26
glycolic acid	0,4	3,24	2,42	117,2	8,48	7,26	111,2			
pyruvic acid	0,1	0,88	0,75	101,0	2,45	2,26	102,9			
malonic acid	0,0	1,77	0,26	674,0	2,29	0,79	291,2	73,93	82,06	90,09
maleic acid	0,0	0,12	0,02	387,4	0,28	0,07	360,6	7,32	40,41	18,02
fumaric acid	0,0	0,34	0,25	131,7	0,88	0,76	115,6			
malic acid	0,1	0,09	0,02	79,4	0,11	0,06	64,3	9,45	29,67	31,59
itaconic acid	0,0	0,03	0,10	2,2	0,03	0,31	3,3	41,03	28,97	141,53
glutaric acid	4,1	4,28	0,11	131,4	4,44	0,33	92,0			
ketoglutaric acid	0,1	0,11	0,02	141,4	0,30	0,07	311,6	8,33	28,68	28,78
glucuronic acid	0,1	0,07	0,02	43,7	0,11	0,06	73,7	8,90	222,65	3,97
galacturonic acid	0,0	0,05	0,02	80,1	0,09	0,06	93,5			
furfural	0,0	0,07	1,13	2,2	0,23	3,39	5,2	72,11	1185,38	6,08
HMF	0,1	1,53	1,15	122,0	3,93	3,46	110,0			

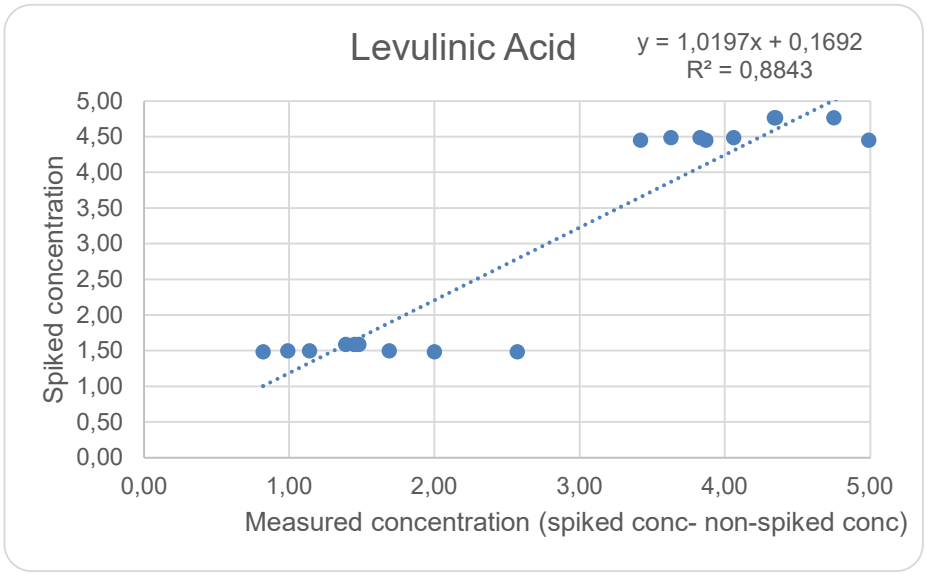
Linearity Plots

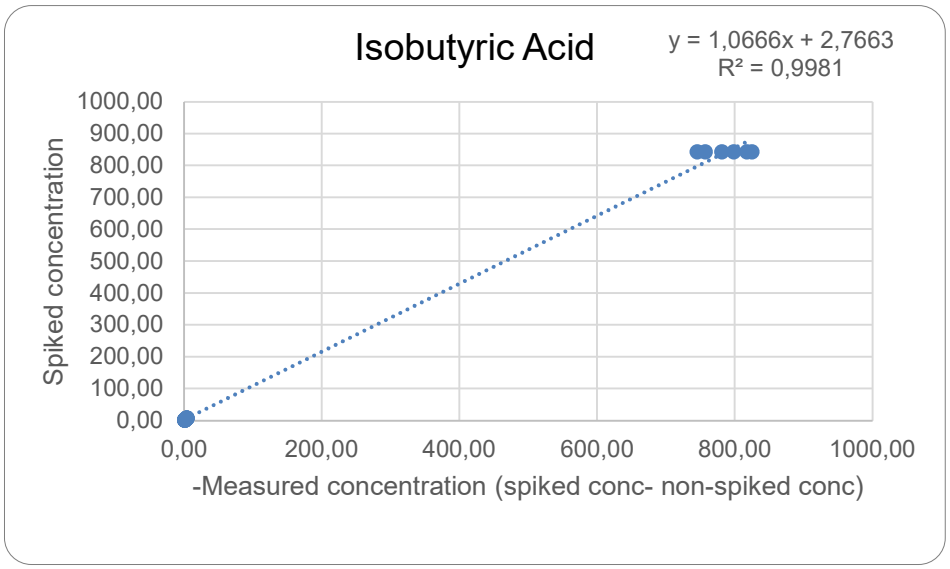


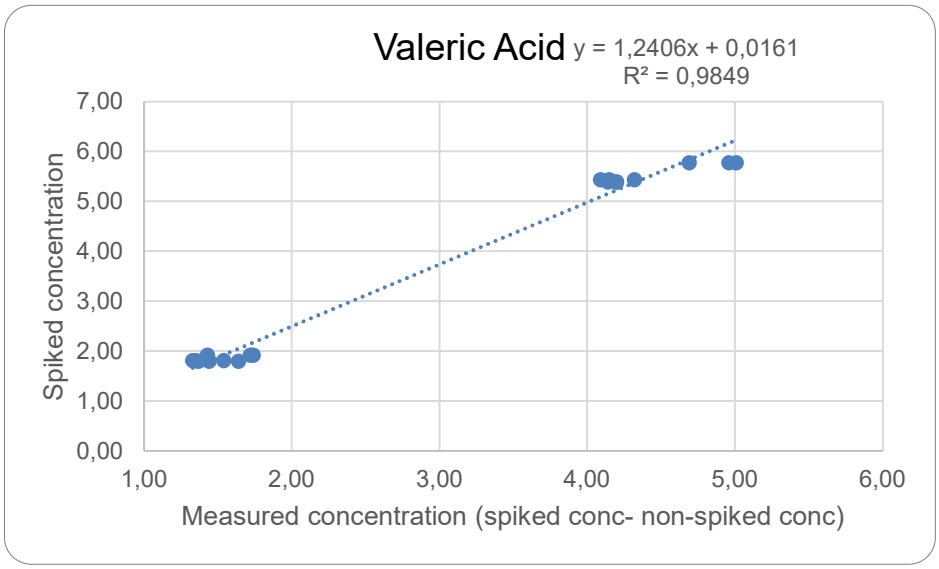


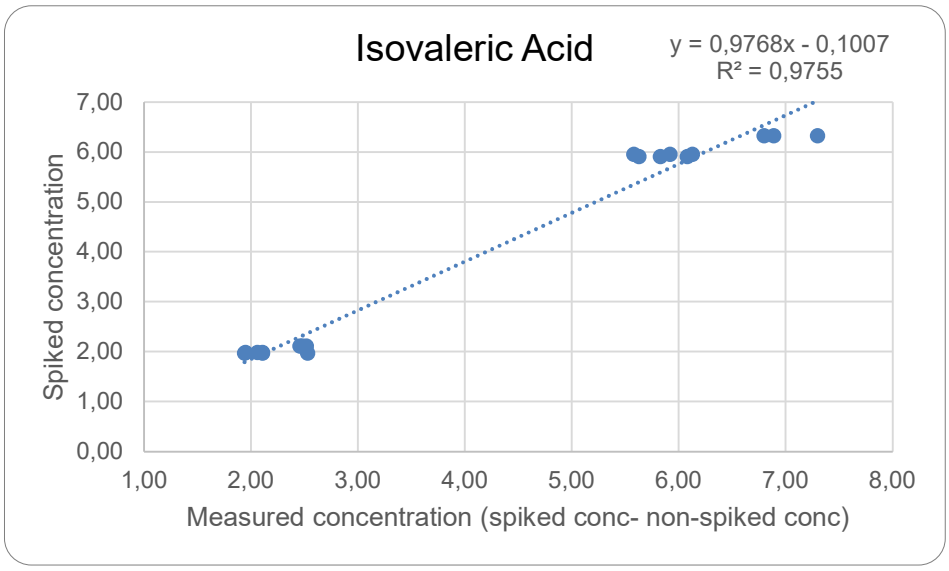


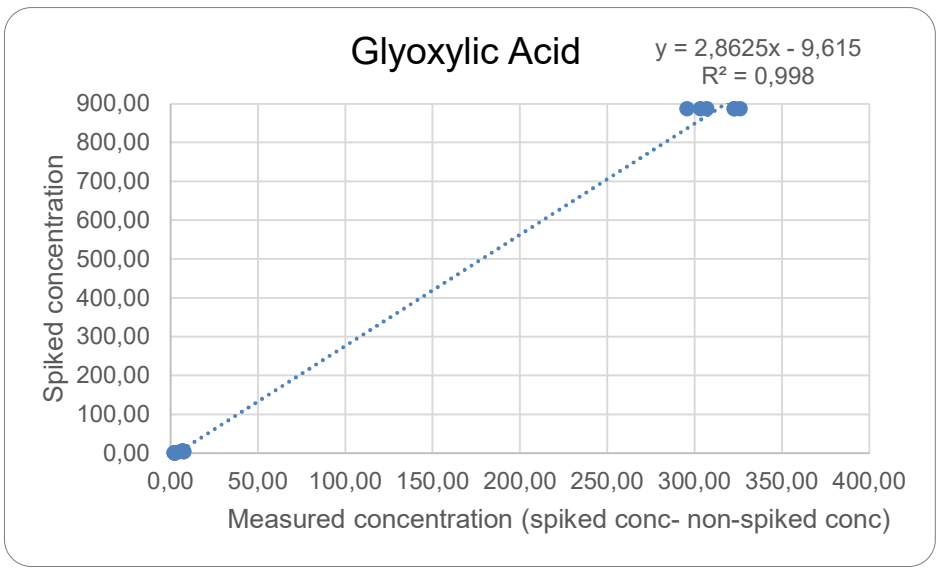

Succinic Acid
y = 0,7707x - 0,0357
R² = 0,9986
Spiked concentration
60,00
50,00
40,00
30,00
20,00
10,00
0,00
0,00 10,00 20,00 30,00 40,00 50,00 60,00 70,00 80,00
Measured concentration (spiked conc- non-spiked conc)

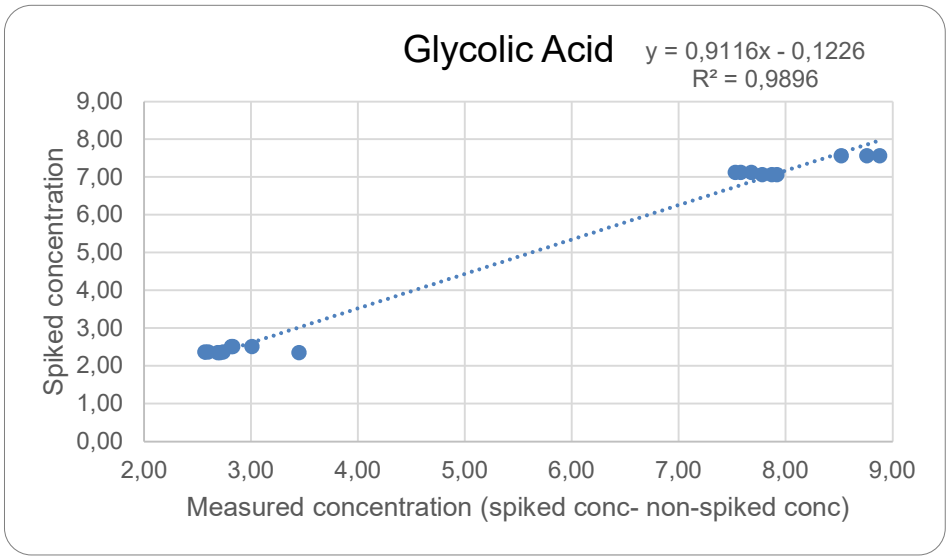

Levulinic Acid
y = 1,0197x + 0,1692
R² = 0,8843
Spiked concentration
5,00
4,50
4,00
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
0,00 1,00 2,00 3,00 4,00 5,00
Measured concentration (spiked conc- non-spiked conc)

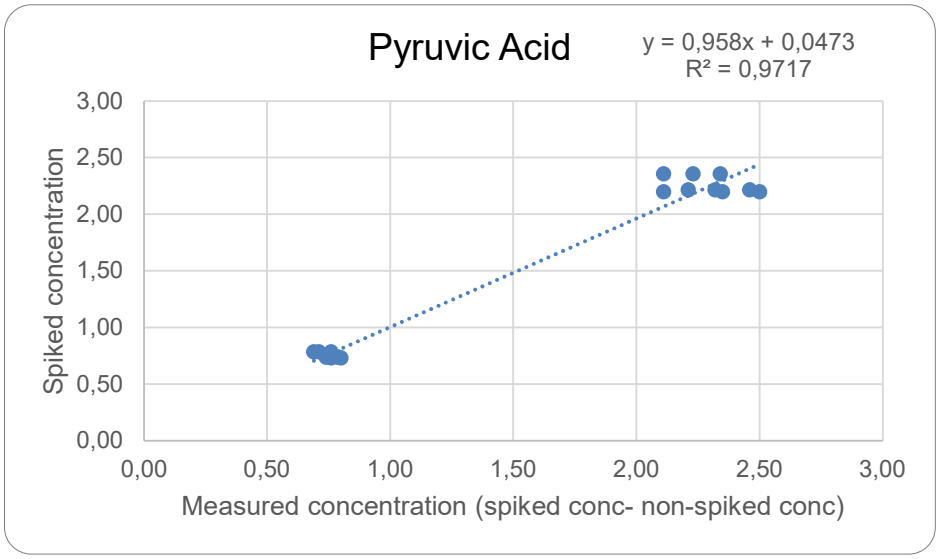

Isobutyric Acid
y = 1,0666x + 2,7663
R² = 0,9981
Spiked concentration
1000,00
900,00
800,00
700,00
600,00
500,00
400,00
300,00
200,00
100,00
0,00
0,00 200,00 400,00 600,00 800,00 1000,00
-Measured concentration (spiked conc- non-spiked conc)

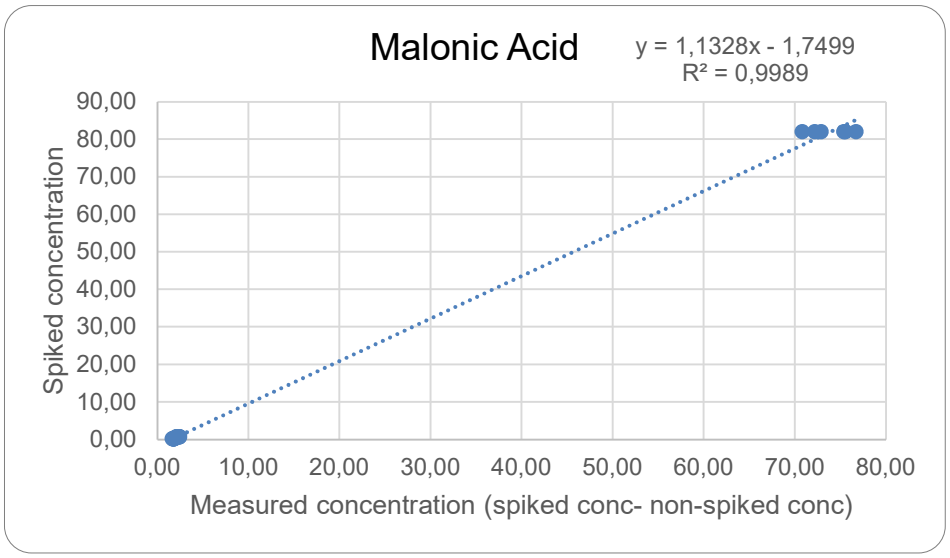

Valeric Acid
y = 1,2406x + 0,0161
R² = 0,9849
Spiked concentration
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
1,00
2,00
3,00
4,00
5,00
6,00
Measured concentration (spiked conc- non-spiked conc)

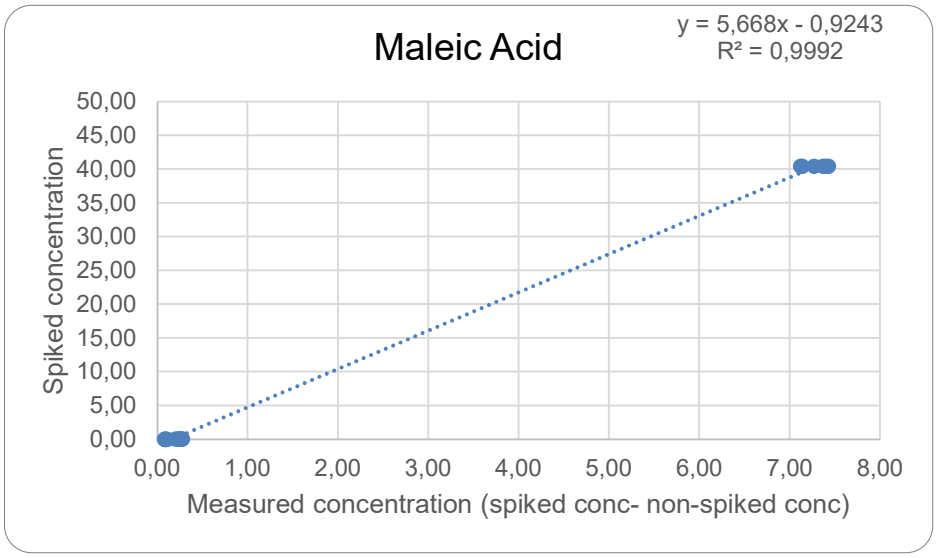

Isovaleric Acid
y = 0,9768x - 0,1007
R² = 0,9755
Spiked concentration
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
1,00
2,00
3,00
4,00
5,00
6,00
7,00
8,00
Measured concentration (spiked conc- non-spiked conc)

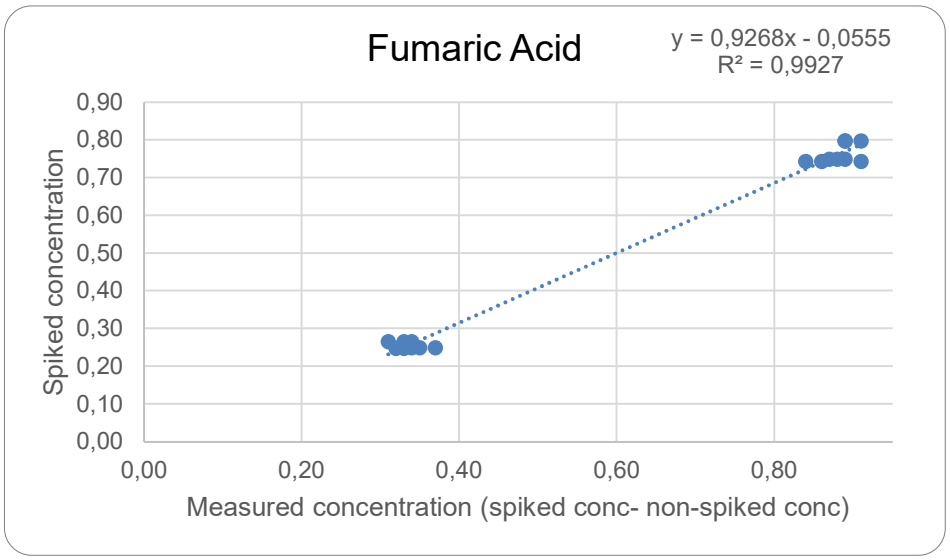

Glyoxylic Acid
y = 2,8625x - 9,615
R² = 0,998
Spiked concentration
900,00
800,00
700,00
600,00
500,00
400,00
300,00
200,00
100,00
0,00
0,00
50,00
100,00
150,00
200,00
250,00
300,00
350,00
400,00
Measured concentration (spiked conc- non-spiked conc)

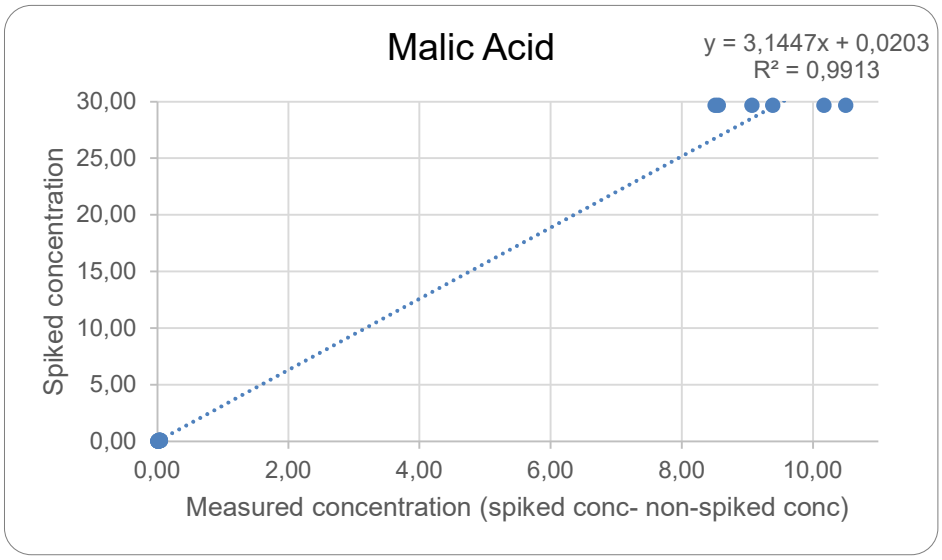

Glycolic Acid
y = 0,9116x - 0,1226
R² = 0,9896
Spiked concentration
9,00
8,00
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
2,00
3,00
4,00
5,00
6,00
7,00
8,00
9,00
Measured concentration (spiked conc- non-spiked conc)

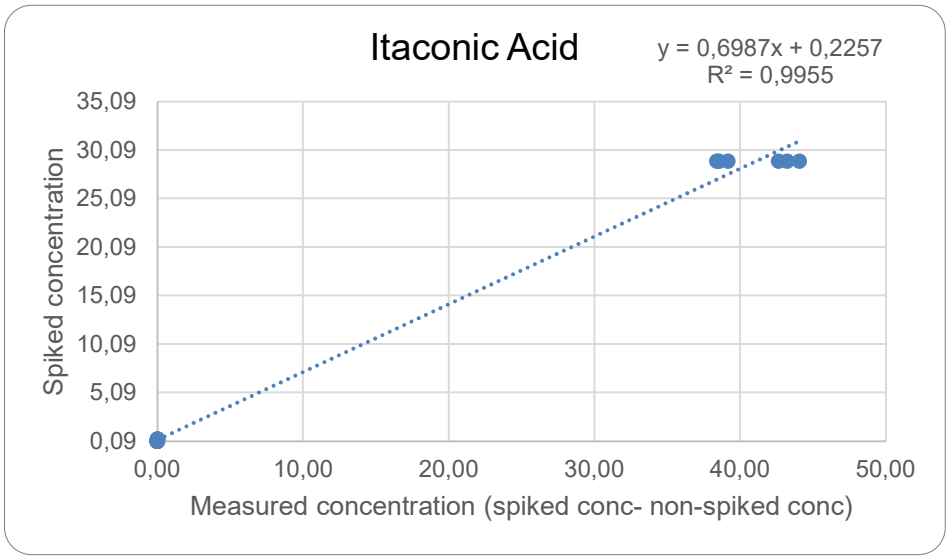

Pyruvic Acid
y = 0,958x + 0,0473
R² = 0,9717
Spiked concentration
3,00
2,50
2,00
1,50
1,00
0,50
0,00
0,00
0,50
1,00
1,50
2,00
2,50
3,00
Measured concentration (spiked conc- non-spiked conc)

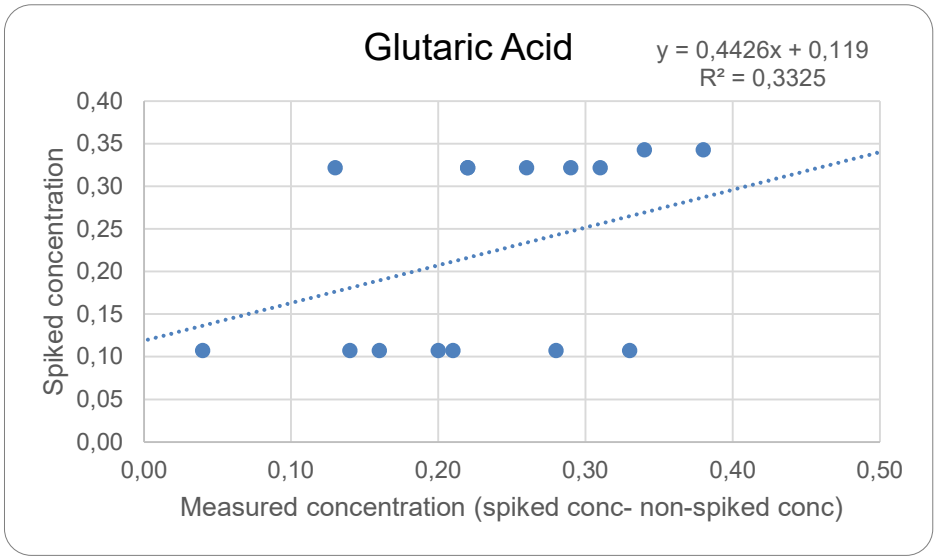

Malonic Acid
y = 1,1328x - 1,7499
R² = 0,9989
Spiked concentration
90,00
80,00
70,00
60,00
50,00
40,00
30,00
20,00
10,00
0,00
0,00
10,00
20,00
30,00
40,00
50,00
60,00
70,00
80,00
Measured concentration (spiked conc- non-spiked conc)

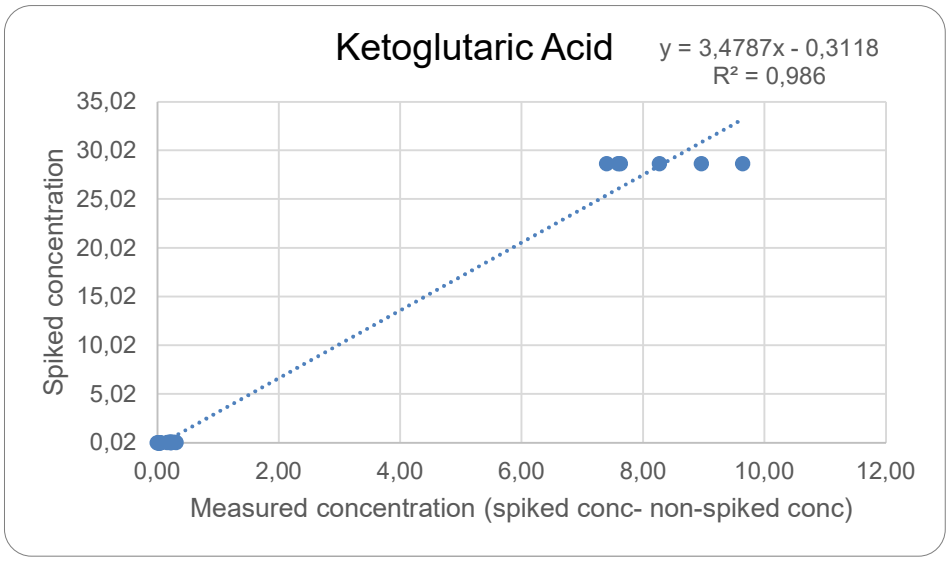

Maleic Acid
y = 5,668x - 0,9243
R² = 0,9992
Spiked concentration
50,00
45,00
40,00
35,00
30,00
25,00
20,00
15,00
10,00
5,00
0,00
0,00
1,00
2,00
3,00
4,00
5,00
6,00
7,00
8,00
Measured concentration (spiked conc- non-spiked conc)

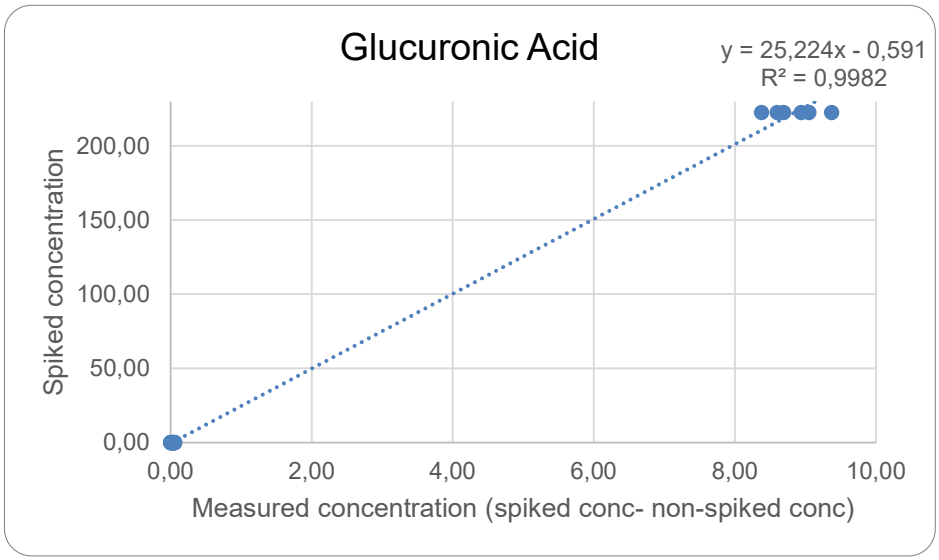

Fumaric Acid
y = 0,9268x - 0,0555
R² = 0,9927
Spiked concentration
0,90
0,80
0,70
0,60
0,50
0,40
0,30
0,20
0,10
0,00
0,00
0,20
0,40
0,60
0,80
Measured concentration (spiked conc- non-spiked conc)

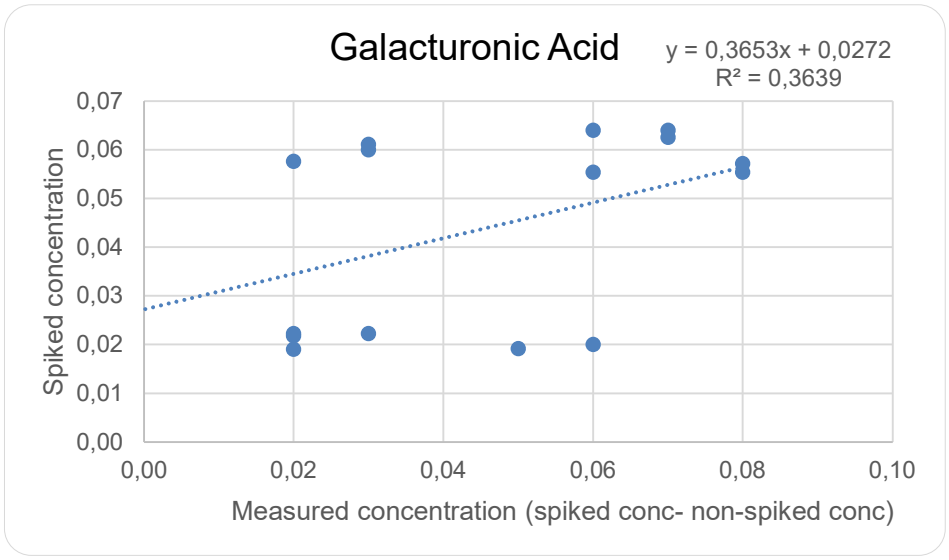

Malic Acid
y = 3,1447x + 0,0203
R² = 0,9913
Spiked concentration
30,00
25,00
20,00
15,00
10,00
5,00
0,00
0,00
2,00
4,00
6,00
8,00
10,00
Measured concentration (spiked conc- non-spiked conc)

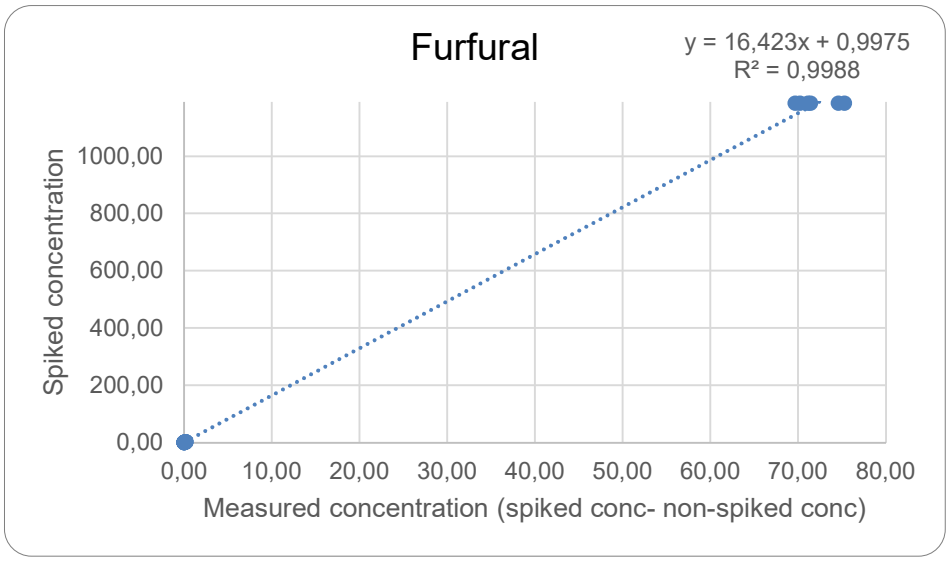

Itaconic Acid
y = 0,6987x + 0,2257
R² = 0,9955
Spiked concentration
35,09
30,09
25,09
20,09
15,09
10,09
5,09
0,09
0,00
10,00
20,00
30,00
40,00
50,00
Measured concentration (spiked conc- non-spiked conc)

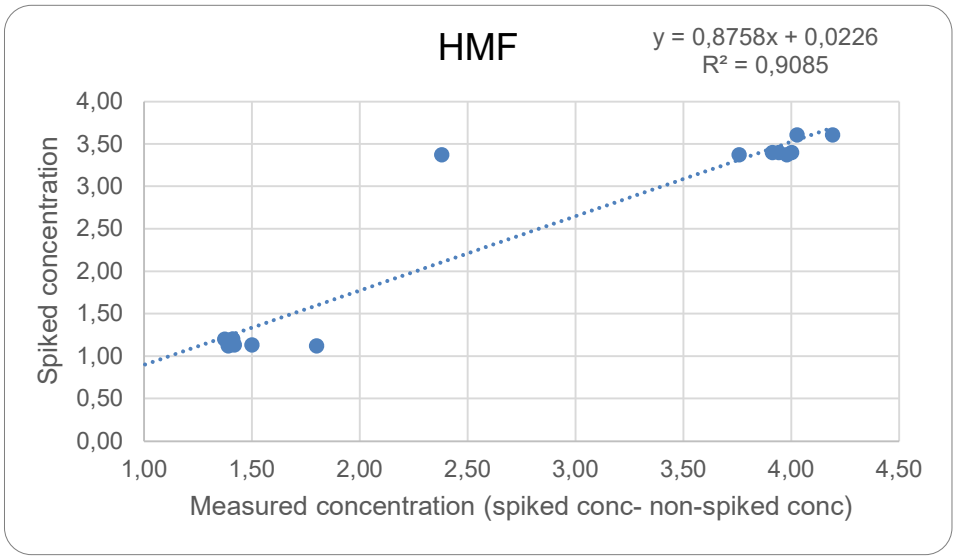

Glutaric Acid
y = 0,4426x + 0,119
R² = 0,3325
Spiked concentration
0,40
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
0,00
0,10
0,20
0,30
0,40
0,50
Measured concentration (spiked conc- non-spiked conc)

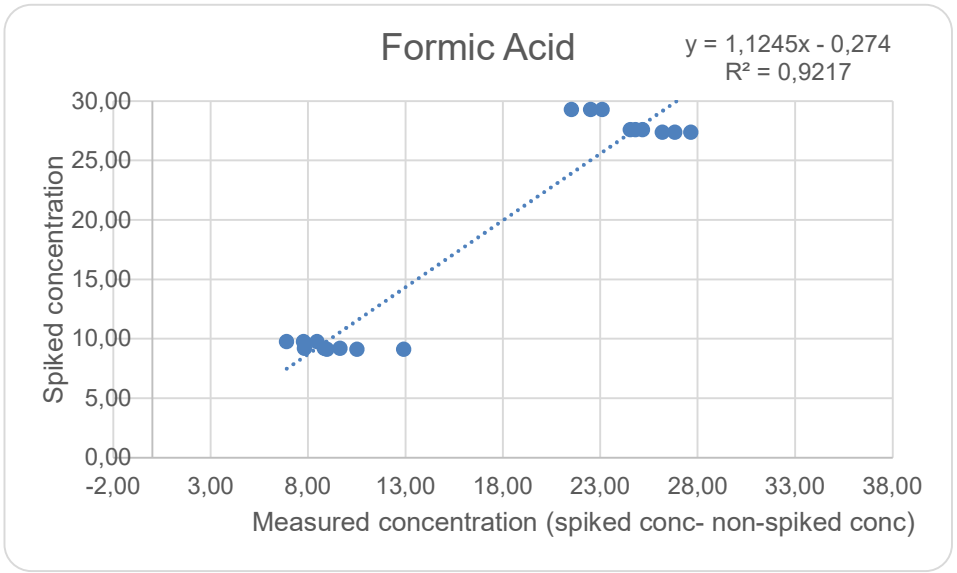

Ketoglutaric Acid
y = 3,4787x - 0,3118
R² = 0,986
Spiked concentration
35,02
30,02
25,02
20,02
15,02
10,02
5,02
0,02
0,00
2,00
4,00
6,00
8,00
10,00
12,00
Measured concentration (spiked conc- non-spiked conc)

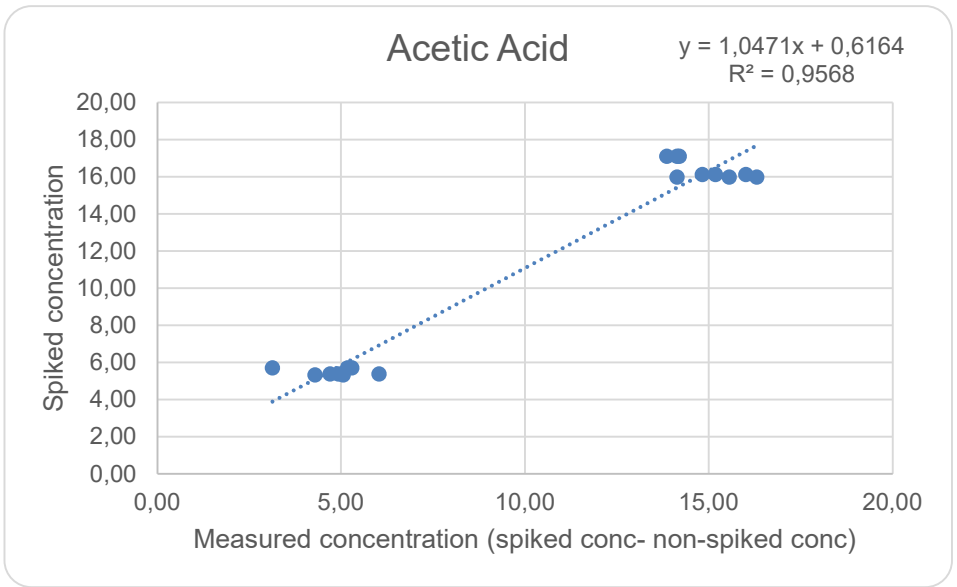

Glucuronic Acid
y = 25,224x - 0,591
R² = 0,9982
Spiked concentration
200,00
150,00
100,00
50,00
0,00
0,00
2,00
4,00
6,00
8,00
10,00
Measured concentration (spiked conc- non-spiked conc)

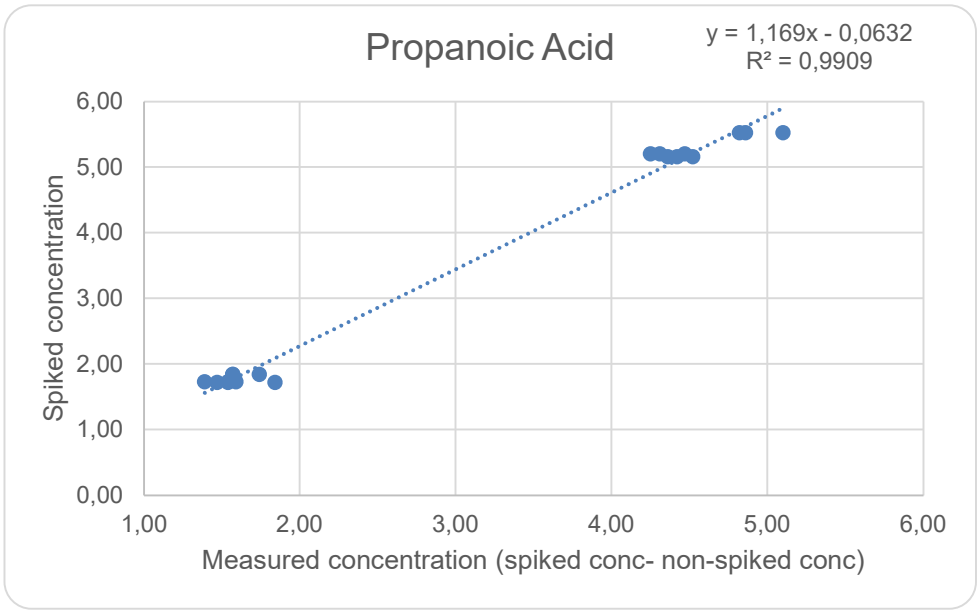

Galacturonic Acid
y = 0,3653x + 0,0272
R² = 0,3639
Spiked concentration
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0,00
0,00
0,02
0,04
0,06
0,08
0,10
Measured concentration (spiked conc- non-spiked conc)


Furfural
y = 16,423x + 0,9975
R² = 0,9988
Spiked concentration
1000,00
800,00
600,00
400,00
200,00
0,00
0,00
10,00
20,00
30,00
40,00
50,00
60,00
70,00
80,00
Measured concentration (spiked conc- non-spiked conc)

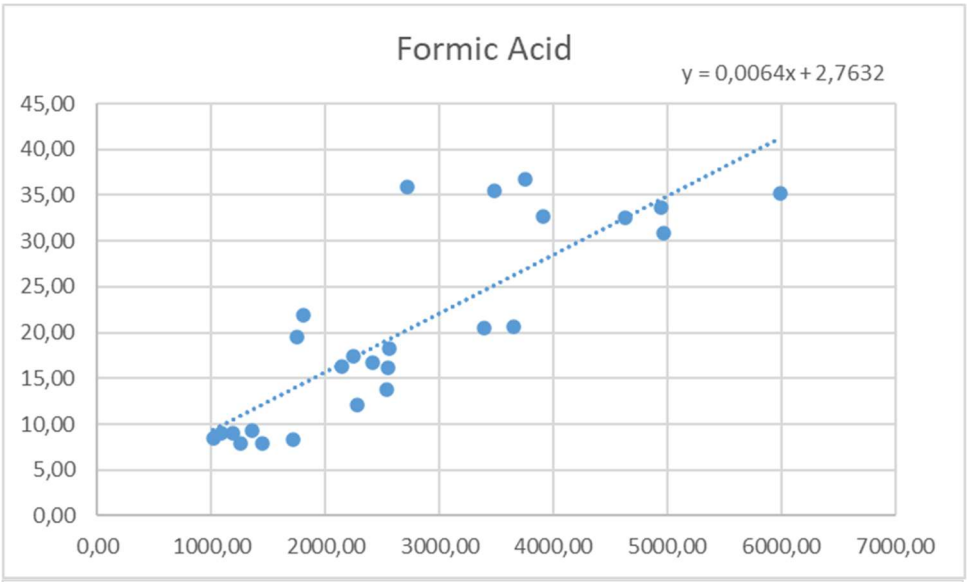

HMF
y = 0,8758x + 0,0226
R² = 0,9085
Spiked concentration
4,00
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
1,00
1,50
2,00
2,50
3,00
3,50
4,00
4,50
Measured concentration (spiked conc- non-spiked conc)

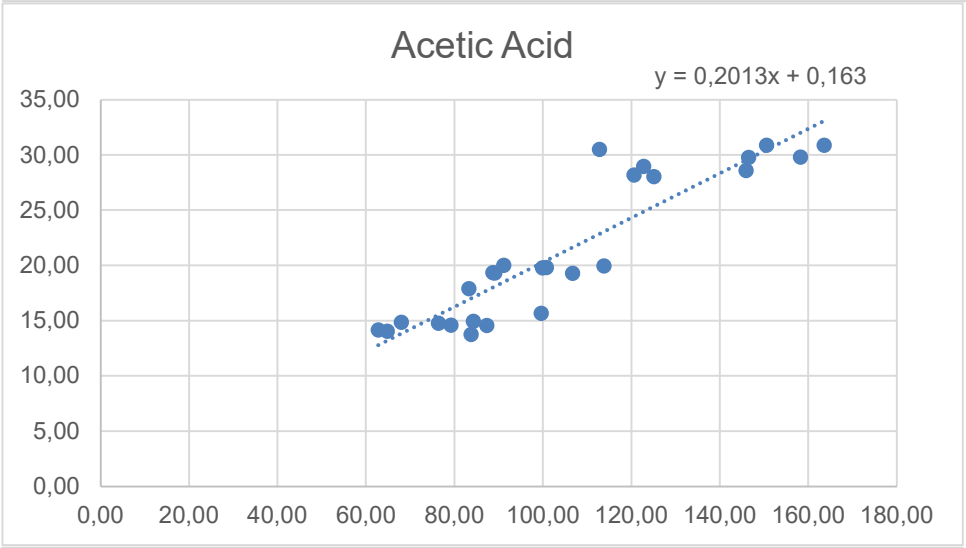

Formic Acid
y = 1,1245x - 0,274
R² = 0,9217
Spiked concentration
30,00
25,00
20,00
15,00
10,00
5,00
0,00
-2,00
3,00
8,00
13,00
18,00
23,00
28,00
33,00
38,00
Measured concentration (spiked conc- non-spiked conc)

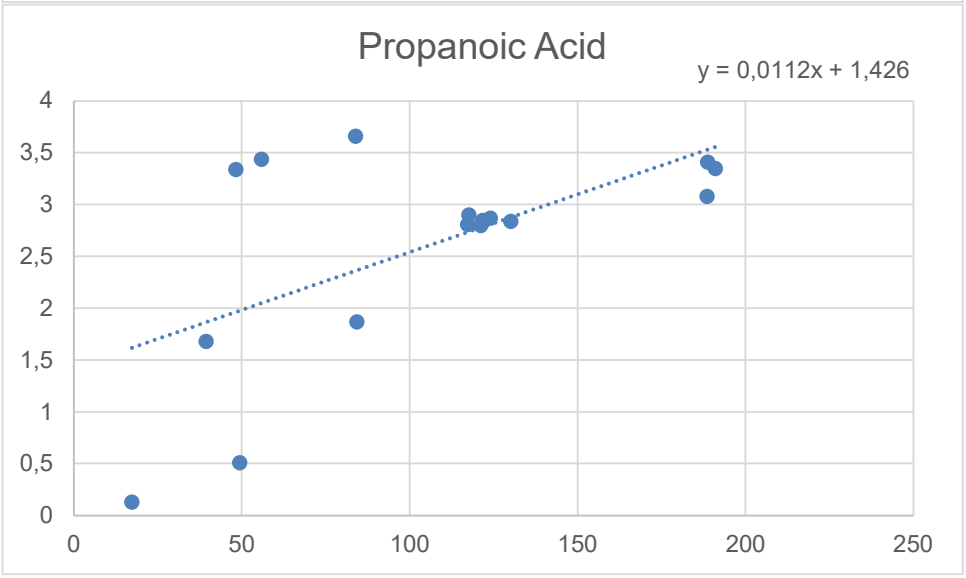

Acetic Acid
y = 1,0471x + 0,6164
R² = 0,9568
Spiked concentration
20,00
18,00
16,00
14,00
12,00
10,00
8,00
6,00
4,00
2,00
0,00
0,00
5,00
10,00
15,00
20,00
Measured concentration (spiked conc- non-spiked conc)

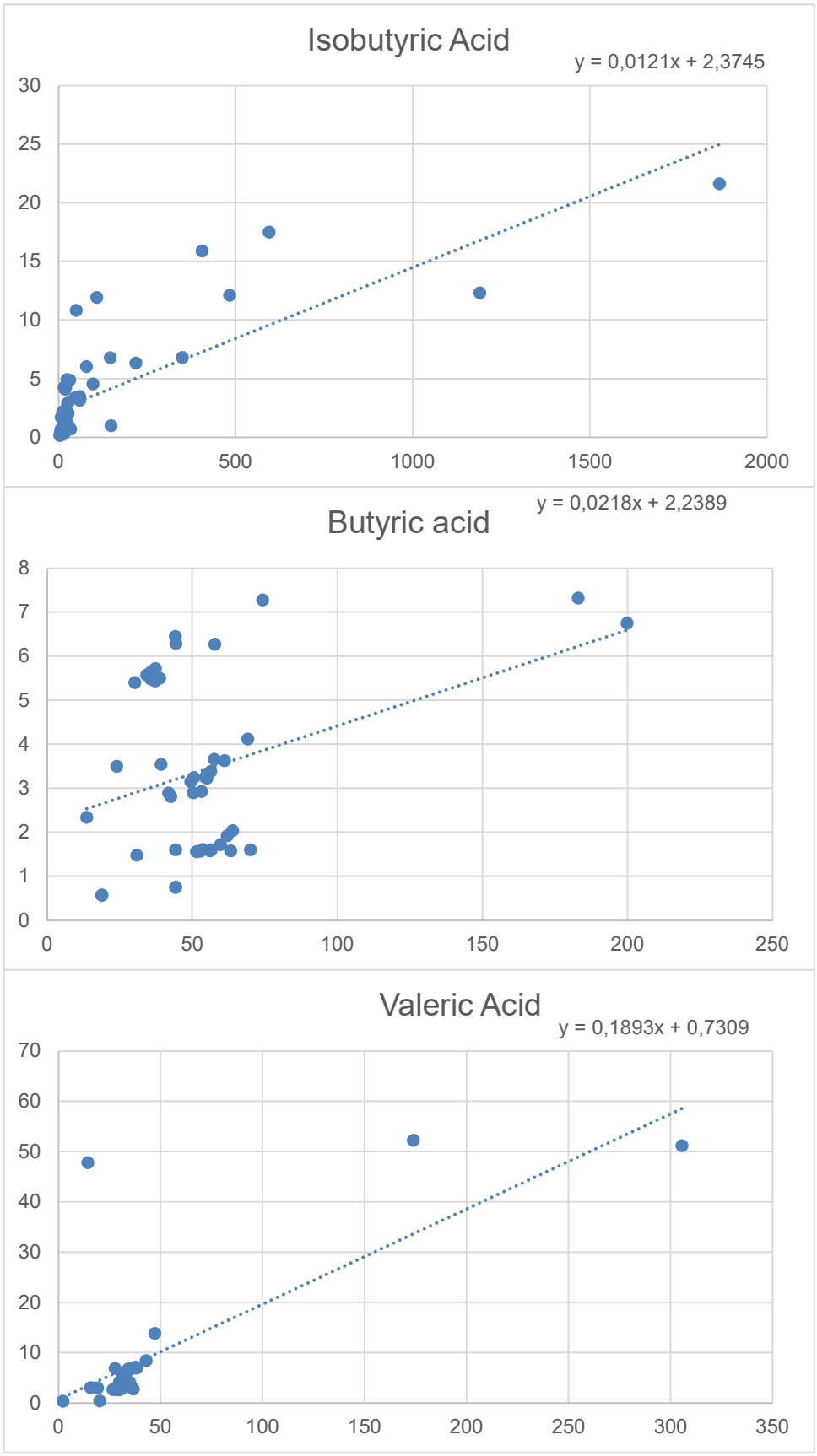

Propanoic Acid
y = 1,169x - 0,0632
R² = 0,9909
Spiked concentration
6,00
5,00
4,00
3,00
2,00
1,00
0,00
1,00
2,00
3,00
4,00
5,00
6,00
Measured concentration (spiked conc- non-spiked conc)

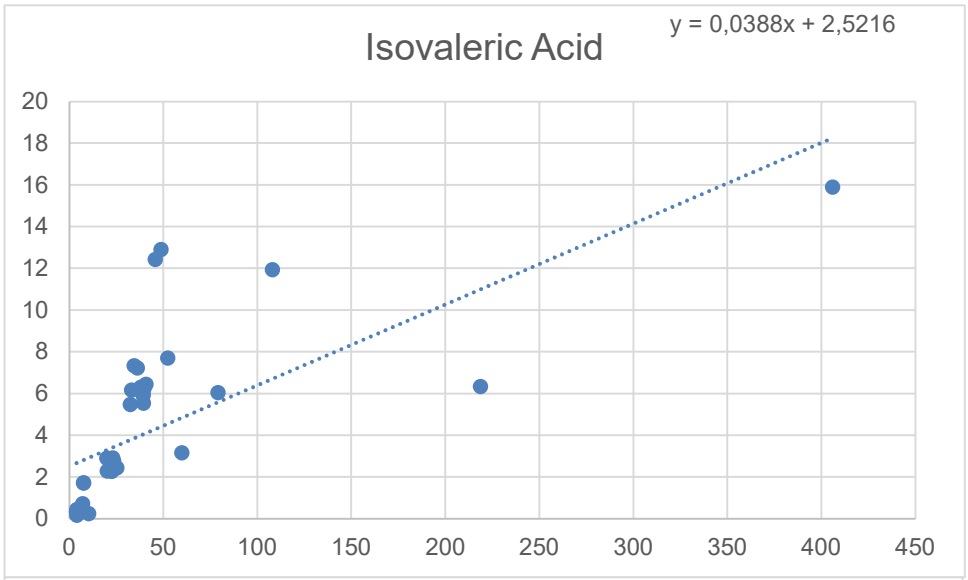
LOD And LOQ Plots

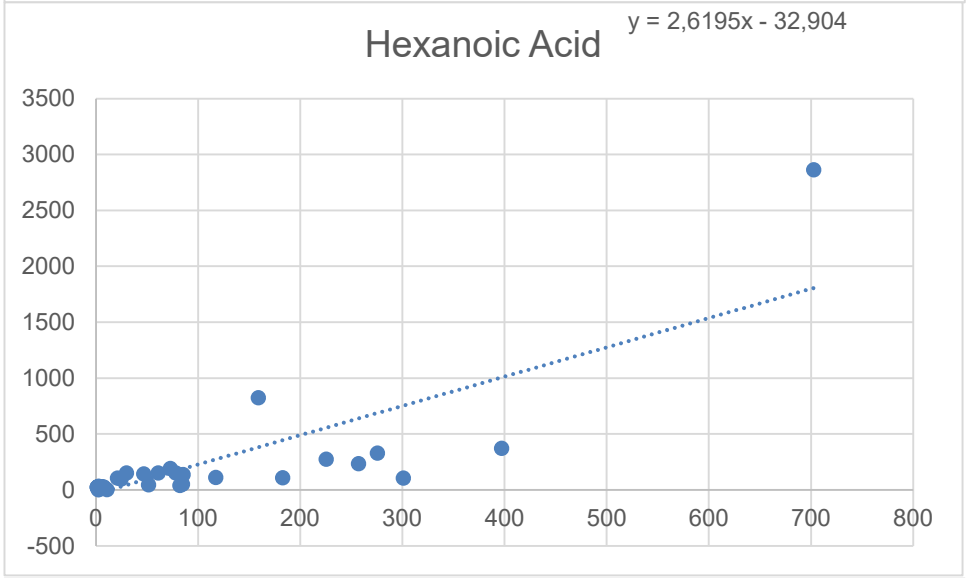


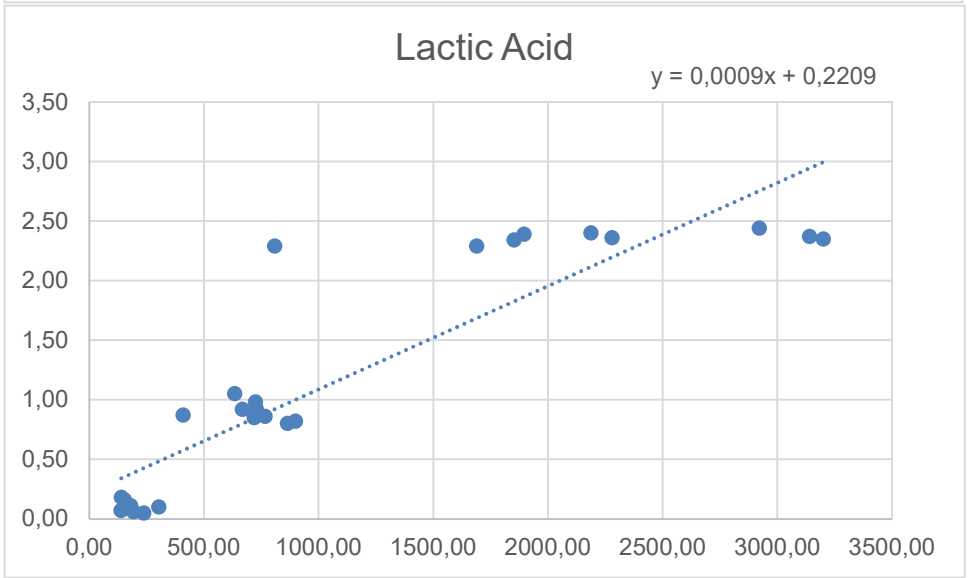


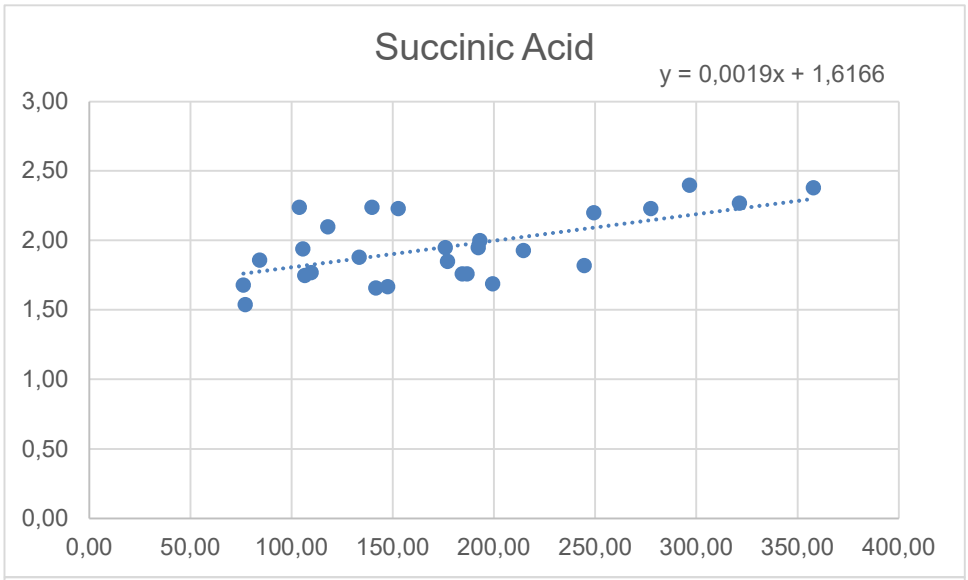


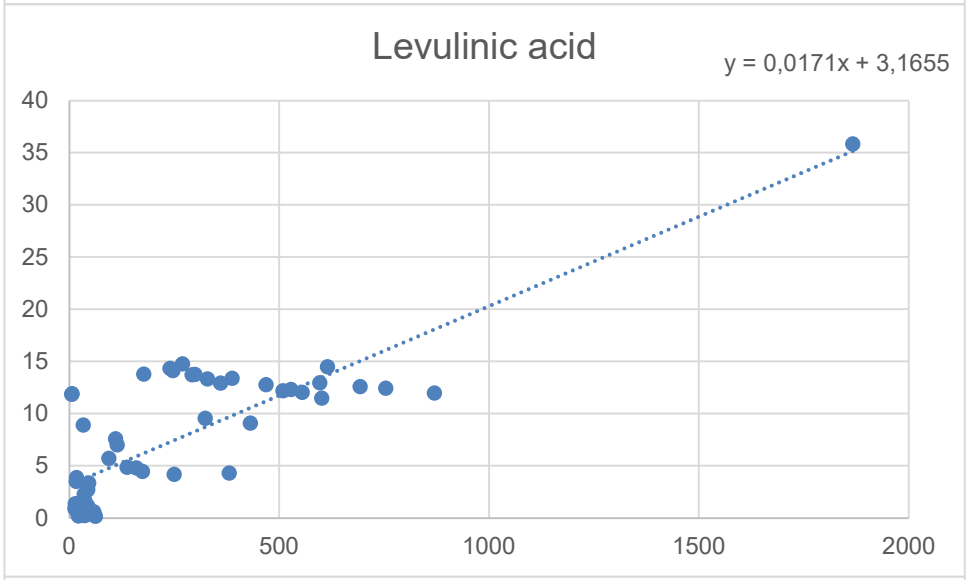

Isobutyric Acid
y = 0,0121x + 2,3745
30
25
20
15
10
5
0
0
500
1000
1500
2000
Butyric acid
y = 0,0218x + 2,2389
8
7
6
5
4
3
2
1
0
0
50
100
150
200
250
Valeric Acid
y = 0,1893x + 0,7309
70
60
50
40
30
20
10
0
0
50
100
150
200
250
300
350

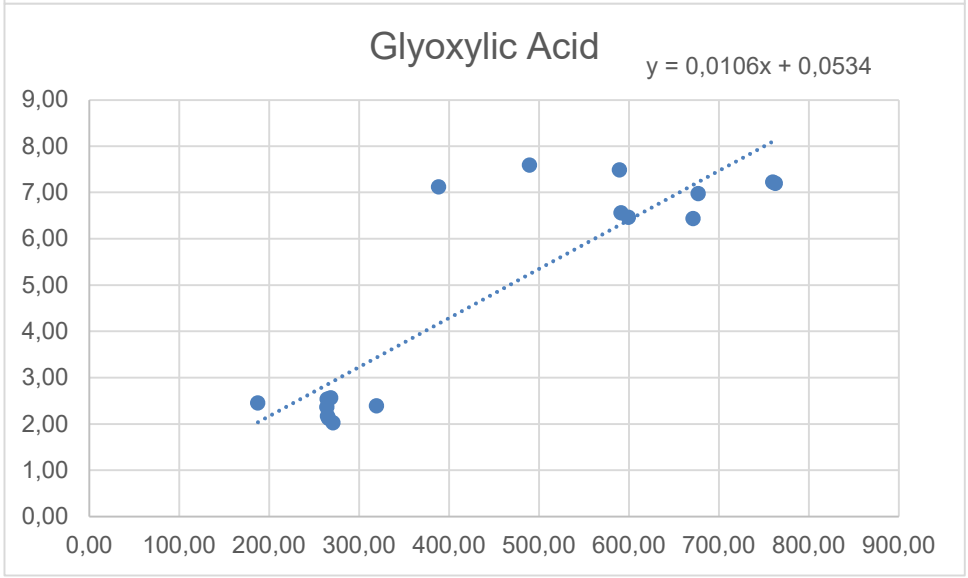

Isovaleric Acid
y = 0,0388x + 2,5216
20
18
16
14
12
10
8
6
4
2
0
0
50
100
150
200
250
300
350
400
450

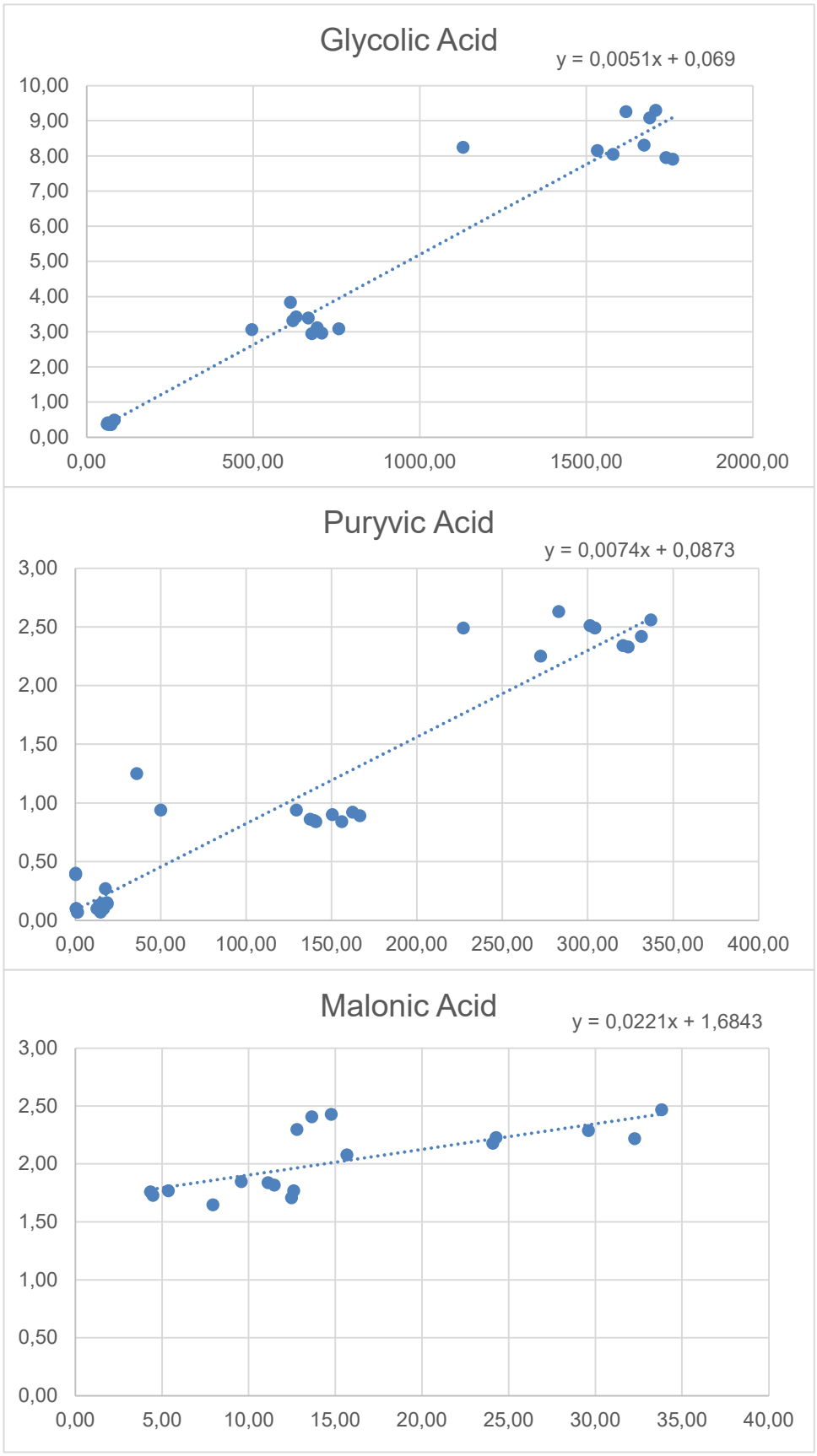

Hexanoic Acid
y = 2,6195x - 32,904
3500
3000
2500
2000
1500
1000
500
0
-500
0
100
200
300
400
500
600
700
800

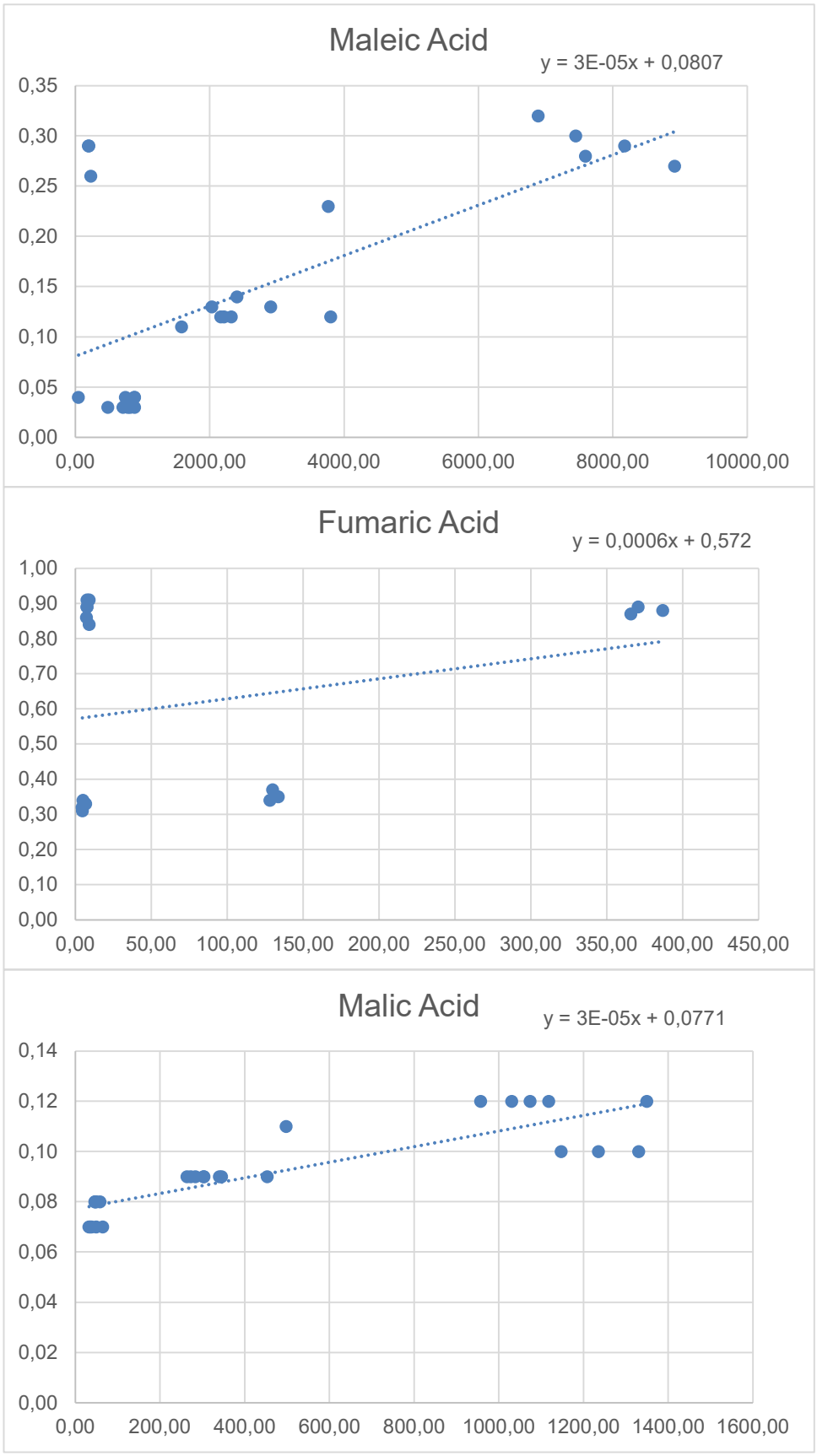

Lactic Acid
y = 0,0009x + 0,2209
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
0,00
500,00
1000,00
1500,00
2000,00
2500,00
3000,00
3500,00

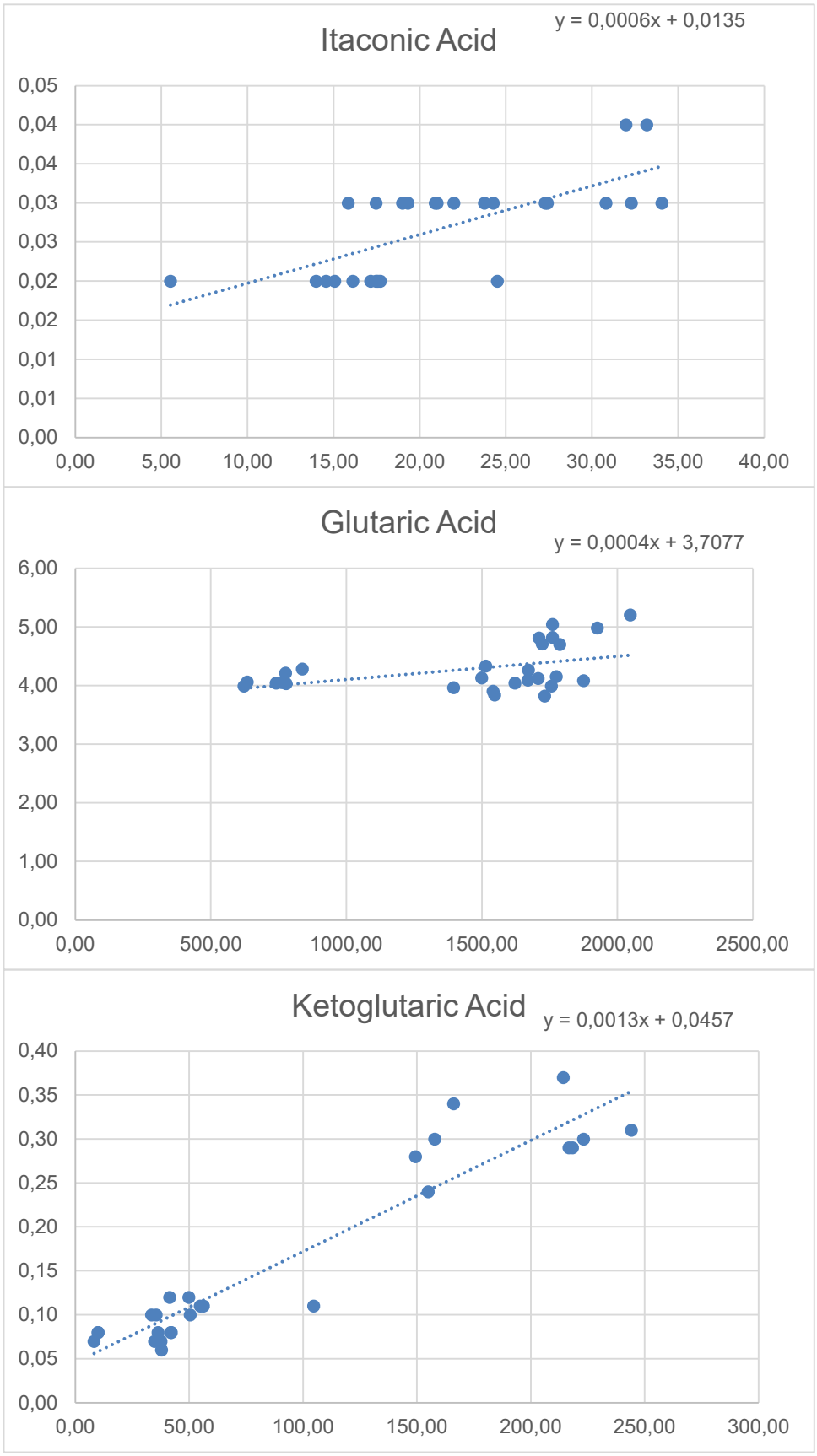

Succinic Acid
y = 0,0019x + 1,6166
3,00
2,50
2,00
1,50
1,00
0,50
0,00
0,00
50,00
100,00
150,00
200,00
250,00
300,00
350,00
400,00

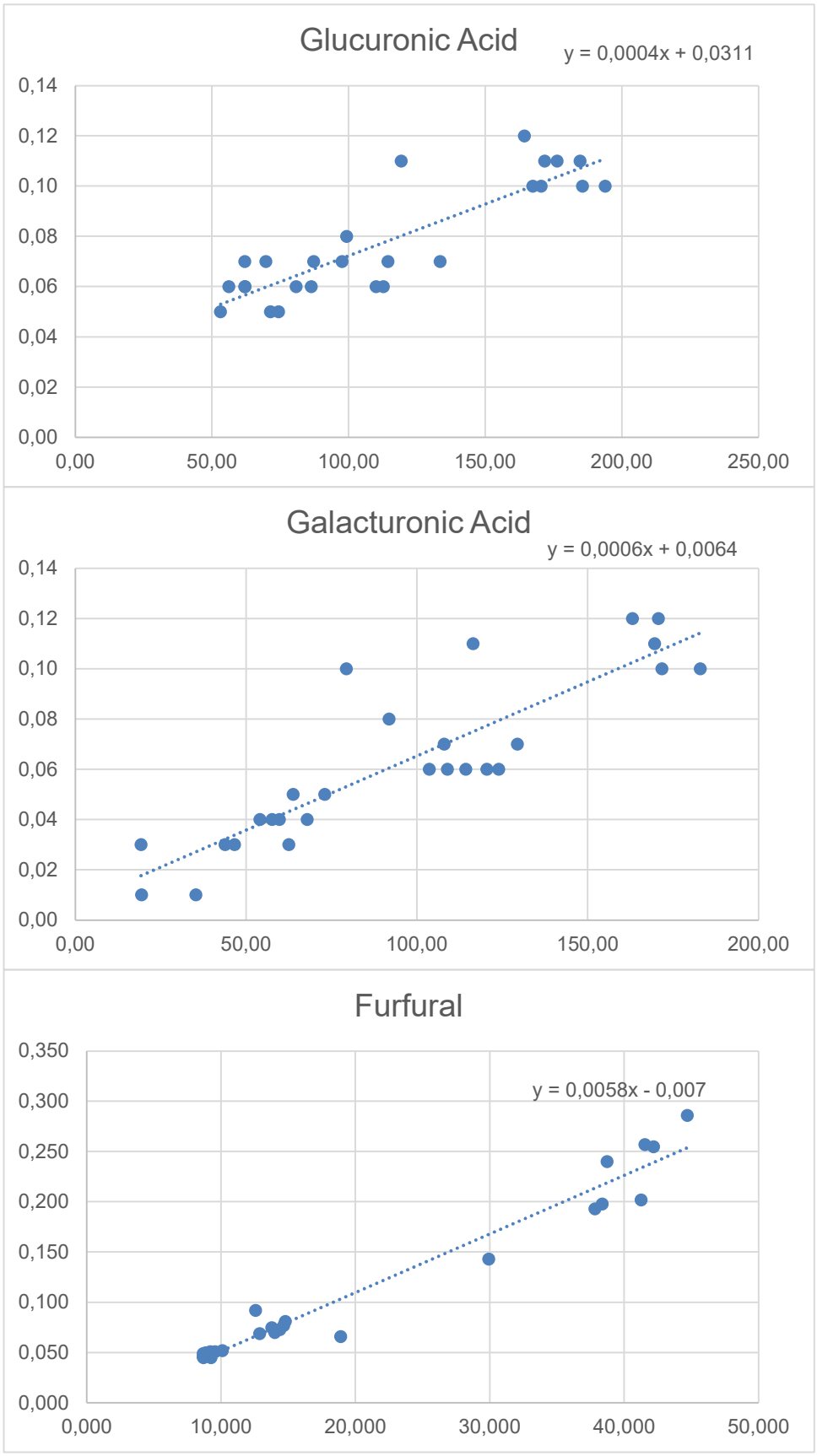

Levulinic acid
y = 0,0171x + 3,1655
40
35
30
25
20
15
10
5
0
0
500
1000
1500
2000

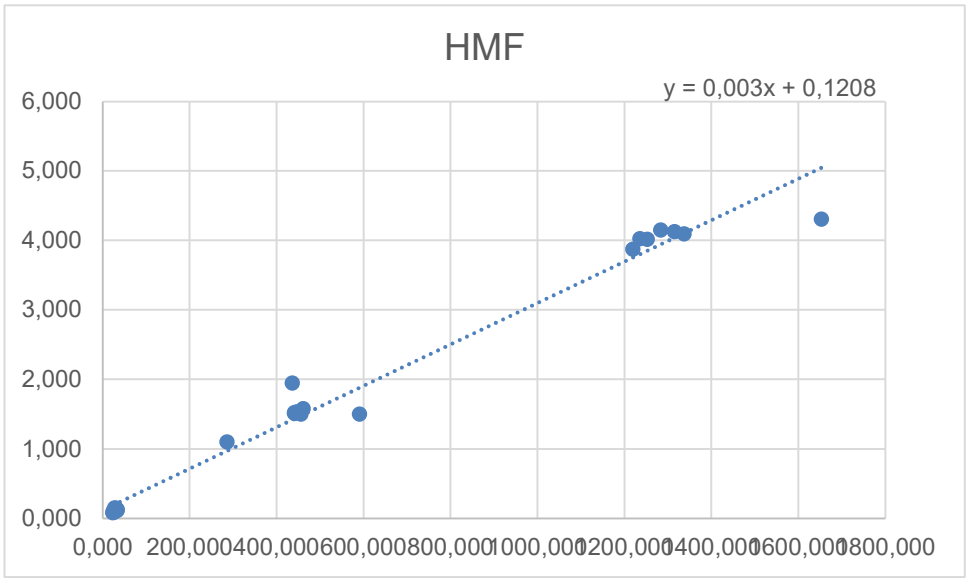

Glyoxylic Acid
y = 0,0106x + 0,0534
9,00
8,00
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
0,00
100,00
200,00
300,00
400,00
500,00
600,00
700,00
800,00
900,00


Glycolic Acid
y = 0,0051x + 0,069
10,00
9,00
8,00
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
0,00
500,00
1000,00
1500,00
2000,00
Puryvic Acid
y = 0,0074x + 0,0873
3,00
2,50
2,00
1,50
1,00
0,50
0,00
0,00
50,00
100,00
150,00
200,00
250,00
300,00
350,00
400,00
Malonic Acid
y = 0,0221x + 1,6843
3,00
2,50
2,00
1,50
1,00
0,50
0,00
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00


Maleic Acid
y = 3E-05x + 0,0807
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
0,00
2000,00
4000,00
6000,00
8000,00
10000,00
Fumaric Acid
y = 0,0006x + 0,572
1,00
0,90
0,80
0,70
0,60
0,50
0,40
0,30
0,20
0,10
0,00
0,00
50,00
100,00
150,00
200,00
250,00
300,00
350,00
400,00
450,00
Malic Acid
y = 3E-05x + 0,0771
0,14
0,12
0,10
0,08
0,06
0,04
0,02
0,00
0,00
200,00
400,00
600,00
800,00
1000,00
1200,00
1400,00
1600,00


Itaconic Acid
y = 0,0006x + 0,0135
0,05
0,04
0,04
0,03
0,03
0,02
0,02
0,01
0,01
0,00
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
Glutaric Acid
y = 0,0004x + 3,7077
6,00
5,00
4,00
3,00
2,00
1,00
0,00
0,00
500,00
1000,00
1500,00
2000,00
2500,00
Ketoglutaric Acid
y = 0,0013x + 0,0457
0,40
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
0,00
50,00
100,00
150,00
200,00
250,00
300,00


Glucuronic Acid
y = 0,0004x + 0,0311
0,14
0,12
0,10
0,08
0,06
0,04
0,02
0,00
0,00
50,00
100,00
150,00
200,00
250,00
Galacturonic Acid
y = 0,0006x + 0,0064
0,14
0,12
0,10
0,08
0,06
0,04
0,02
0,00
0,00
50,00
100,00
150,00
200,00
Furfural
y = 0,0058x - 0,007
0,350
0,300
0,250
0,200
0,150
0,100
0,050
0,000
0,000
10,000
20,000
30,000
40,000
50,000


HMF
y = 0,003x + 0,1208
6,000
5,000
4,000
3,000
2,000
1,000
0,000
0,000
200,000
400,000
600,000
800,000
1000,000
1200,000
1400,000
1600,000
1800,000