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Kaumera Methods Manual

A Comprehensive Guide to Lab Extraction and Characterization Methods for Extracellular Polymeric Substances (EPS)

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EXECUTIVE SUMMARY

Kaumera are extracellular polymeric substances (EPS) extracted from excess aerobic granular sludge from Nereda® wastewater treatment plants. Kaumera exhibits significant market potential across diverse applications, fostering rapid research and business development. Furthermore, it will begin to be extracted from numerous installations worldwide. This calls for standard methods as analogue to (waste)water and sludge characterization.

Due to lack of standardization, stakeholders are currently using different extraction and characterization protocols, impeding the development of a more uniform product and comparison of results across research studies. To address this, this report compiles the standard protocol for Kaumera extraction in the laboratory and for on-site and lab characterization to be used by researchers, the public Dutch water authorities, and the private industry. The procedures detailed in this document are in accordance with EPS research conducted at TU Delft and methodologies employed in Kaumera production facilities.

This report aids in monitoring Kaumera characteristics worldwide and for optimizing the extraction process (including up and downstream processing). This will help maximize repeatability, interoperability, and quality and therefore accelerate business and research development, paving the way to develop a product that meets the needs of the end-users. Through the widespread adoption of this manual, our aim is to foster greater coordination and collaboration among stakeholders, thereby expediting the realization of Kaumera's full potential.

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1. INTRODUCTION

Kaumera® is the product name for the extracellular polymeric substances (EPS) extracted from excess aerobic granular sludge obtained from the Nereda® technology. This polymer extraction process is viable and the product harbors significant potential for diverse market applications (STOWA, 2019). One of the most promising short-term applications for these extracted polymers is in the agricultural and horticultural sector, where they can be used as a combined biostimulant and water absorber for coating fertilizers and seeds. Beyond these applications, there is a wide range of potential uses on the horizon, ranging from fire-resistant composite materials to water-repellent coatings for paper and cardboard (Chen et al., 2021). Furthermore, biomaterials based on EPS provide a sustainable alternative to synthetic polymers. Potentially, 5-10 kilograms of dry Kaumera per person per year can be produced in the wastewater treatment plants, which roughly equals 20% of the plastic demand. Kaumera also has the potential to serve as a replacement for alginate, a mass-produced substance derived from brown seaweed.

Kaumera is primarily composed of glycoprotein polymers in their acidic form, with a molecular weight range of 50-150 kDa (S. Picken, personal communication, October 26, 2023). These polymers are suspended in a liquid phase (water), which contains organic and inorganic compounds like humic substances, organic monomers, and ions. To improve logistical convenience and prolong shelf life, Kaumera can be dried at temperatures below 60°C. Depending on the intended application, it might be advantageous to remove dissolved compounds present in Kaumera using methods like washing or dialysis. However, it is essential to emphasize that any process or treatment applied to Kaumera—whether involving washing, dialysis, or variations in temperature, pH, and conductivity—can cause alterations in the characteristics and behavior of the polymer. These alterations impact multiple aspects, including the glycoprotein's three-dimensional structure, polymer charge, and the degree and type of crosslinking. Therefore, in the light of reproducibility, these factors should be consistent throughout the sample preparation and characterization methods. Regardless of the specific procedures used, it is important to consider and thoroughly document these steps, enabling future studies to compare measurements effectively.

The composition and behavior of Kaumera are influenced by certain factors, such as the choice of sludge material and the extraction method used. Kaumera can be obtained from diverse sources, such as full-scale granules, flocculant sludge, or a mixture of both. Sludge type can either be municipal or industrial. Moreover, multiple extraction methods exist, differing in initial sludge concentration, base and acid used, and pH conditions. These differences can lead to the formation of entirely distinct polymers. In addition, there are multiple methods that can be used to characterize Kaumera in both laboratory and on-site settings, making it challenging to compare results.

Recognizing the significant market potential of Kaumera and its increasing extraction from various installations worldwide, there is a pressing need to standardize these methods. This document consolidates the standard laboratory extraction and characterization methods to be used by operators of Kaumera extraction facilities, researchers, and companies interested in obtaining Kaumera. In summary, Chapter 2 and 3 are tailored for laboratory users, covering sludge sampling and Kaumera extraction on a lab scale, respectively. Chapter 4 provides recommendations for storage to enhance shelf life, and Chapter 5 discusses general sample preparation methods for laboratory analysis. Furthermore, Chapter 6 encompasses a diverse range of characterization methods employed in the laboratory, while Chapter 7 outlines the current protocols used for characterization in Kaumera installations. Additionally, Appendix A includes a ‘Kaumera sample form’ designed to facilitate the recording of essential information regarding the sample's origin, extraction steps, and sample preparation methods. The purpose of this form is to streamline result comparison and maintain a comprehensive record of procedures. The remaining appendices contain detailed step-by-step procedures corresponding to the methodologies outlined in Chapters 5, 6, and 7.

By encouraging the adoption of these methods, this document aims to promote consistency and facilitate meaningful cross-comparisons. Although efforts have been made to provide broadly applicable methods, samples with exceptionally high or unique concentrations, compositions, or characteristics may pose challenges that prevent the direct application of these methods. Additionally, research objectives may vary in certain cases. As a result, adjusting a procedure to suit specific circumstances may become necessary. Whenever a procedure is modified, the analyst should clearly document the nature of the modification in the results report.

Anticipating future optimization or modification of methods to meet specific goals, this document will be periodically updated to integrate new advancements or modifications. This will ensure that changes are tracked and consistent procedures are maintained across all stakeholders. If you have an inquiry on the manual or want to propose an update please contact Yuemei Lin - Professor EPS at TU Delft - Yuemei.Lin@tudelft.nl or Mark van Loosdrecht - Professor in Environmental Biotechnology at TU Delft - M.C.M.vanLoosdrecht@tudelft.nl.

2. SLUDGE SAMPLING

In Nereda installations, two types of waste sludge are generated: (1) the sludge removed in each cycle, referred to as aerobic granular sludge selection discharge or selection spill (AGS-SD) (Guo et al., 2020). This sludge is more flocculent and settles at a lower velocity than the aerobic granules. It also contains particles (cellulose, primary sludge, etc.) from the influent that do not settle fast enough. Its removal introduces a biological selection pressure favoring the faster-settling larger granules. Due to its removal in each cycle, this waste aerobic granular sludge experiences a shorter retention time than the granules (Ali et al., 2019); (2) the excess granular sludge resulting from biomass growth, removed to prevent excessively high biomass concentrations in the reactor, known as solid retention time control of the AGS (AGS-RTC) (Guo et al., 2020). This manual exclusively defines "Kaumera" as being extracted from the selection spill sludge. Nevertheless, EPS extraction can be carried out from various AGS fractions, such as exclusively granules, only the flocculant fraction, solely granules of specific sizes, and various combinations or mixtures of these fractions. However, extracting EPS from a different source may lead to distinct composition and properties. Future adjustments may be considered based on the desired product characteristics for a specific application.

There are also other factors that can affect the properties and composition of Kaumera. Nereda installations treat either municipal or industrial wastewater, resulting in variations in Kaumera characteristics depending on the type of wastewater processed. For instance, Kaumera sourced from industrial sludge may exhibit significantly lower concentrations of heavy metals compared to Kaumera derived from municipal sludge (STOWA, 2019). Industrial streams may also contain various (high) concentrations of carbon sources potentially leading to the enrichment of different bacterial populations compared to municipal sewage. In turn, this might yield Kaumera with characteristics that differ significantly. In addition, factors such as the operational parameters of the Nereda installation (e.g., SRT, iron dosing), the application of polyelectrolytes before sludge sampling, and the storage conditions of the sludge prior to extraction—whether in a sludge buffer or laboratory—can impact the characteristics and behavior of the resulting Kaumera product. It is advisable to minimize storage time for the latter. Therefore, maintaining comprehensive documentation of these parameters is necessary to ensure accurate records and facilitate research comparison.

3. LAB EXTRACTION PROTOCOL

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3.1. INTRODUCTION

In aerobic granular sludge (AGS), microorganisms produce a substantial amount of highly hydrated extracellular polymeric substances (EPS) (Flemming et al., 2010). This production serves to create a hydrogel matrix in which the microorganisms can be self-immobilized (Seviour et al., 2009; Flemming et al., 2010; Seviour 2012). As these polymeric substances interact with each other through electrostatic forces, hydrogen bonds, attractive ionic forces, and biochemical reactions (Flemming et al., 2010), a dense and compact tertiary network structure is formed (Felz et al., 2016). The polymers within EPS that contribute to the formation of hydrogels (Seviour et al., 2009; Lin et al., 2013) and the tertiary network structure are specifically termed structural EPS, representing a subset of the overall EPS (Felz et al., 2016). To solubilize these polymers, it is necessary to disrupt the interactions that bind them together. This disruption is achieved through extraction methods designed to target specific types of EPS bonds within the matrix (D'Abzac et al., 2009).

Various techniques are available for the extraction of EPS. Harsh extraction methods, which may involve physical approaches like heating, centrifugation, and sonication, as well as chemical methods such as alkaline extractions, chelators, and detergents are required (Felz et al., 2016). The selection of the extraction method, including the choice of chemicals, their concentrations, pH conditions, and the sequence of events, not only impacts the total quantity of recovered polymers but also influences the composition of the extracted material. Depending on the characteristics of the sludge and the targeted EPS, a method is selected.

In the production of Kaumera in the full-scale installations (see Figure 1) and EPS research at TU Delft, the chosen approach includes introducing an alkaline reagent to the sludge, coupled with heat and mixing to improve solubilization. Following this, a centrifugation step separates the alkaline centrate containing the EPS from the discarded alkaline solid residue. The precipitation of structural EPS involves the introduction of an acid, followed by another centrifugation step, during which the acidic structural EPS gel is isolated from the acidic centrate. Within this methodology, there still exist several extraction methods that have been applied, which vary in the sludge type and concentration, base and acid used, pH conditions, and centrifugation and mixing setups. Through consensus, the lab protocol outlined in Bahgat et al. (2023) was selected as the standard for Kaumera research, as it closely mirrors the full-scale production process of Kaumera. For this protocol, it is important to note that replicating the centrifugation and mixing steps of the full-scale production process proved challenging, mainly due to differences in the equipment.

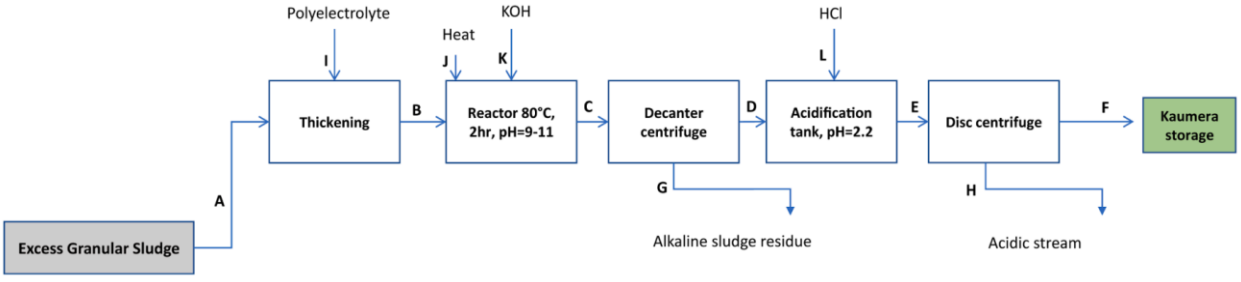

Figure 1: Kaumera extraction process from excess aerobic granular sludge in Epe installation (Bahgat et al., 2023).

In TU Delft, the extraction protocol of Felz et al. (2016) is commonly used in EPS research (Table 1). However, there was a significant dilution difference between the protocol of Felz et al. (2016) and what can be found in large-scale installations. This dilution affected the concentrations of all elements in the liquid streams. Moreover, when employing the undiluted protocol from Bahgat et al. (2023), a new gel layer emerged during the alkaline step—something not observed previously (Figure 2). This leads to the hypothesis that dilution might selectively fractionate the polymer, possibly based on molecular weight. The Kaumera yield remained the same in both protocols (diluted and undiluted), indicating that the gel layer observed during the alkaline step was transferring to the acidic supernatant and not being recovered. This suggests a potential opportunity for recovery.

Using the extraction protocol of Bahgat et al. (2023) for Kaumera research is recommended. In the event that modifications are necessary to achieve a particular objective, it is important to document these changes. However, it is essential to make any alterations deliberately and after careful consideration. In the future, extractions might be optimized depending on the end-use application.

Table 1: Differences between Felz et al. (2016) laboratory extraction, Epe and Zutphen demo scale extractions, and the established new modified protocol (Bahgat et al., 2023).

Parameters	Original (diluted) laboratory protocol (Felz et al., 2016)	Epe/Zutphen demonstration scale practice	New modified (undiluted) laboratory protocol (Bahgat et al., 2023)
Sludge used	Granules ≥2mm	Excess granular sludge	Excess granular sludge
		(small granules + flocs)	(small granules + flocs)
Sludge concentration	<0.8 w/v%	5 w/v%	5 w/v%
Base used	0.5% (w/v) Sodium carbonate	25% (w/v) Potassium hydroxide	25% (w/v) Potassium hydroxide
Base addition	No pH control	pH-controlled 9–11	pH-controlled 9–11
Alkaline extraction	80°C, 30 min	80°C, 2 h	80°C, 2 h
Acid used	1 M Hydrochloric acid	9.5 M Hydrochloric acid	9.5 M Hydrochloric acid
	pH 2–4	pH 2–4	pH 2–4
Centrifugation	4°C (4000 x g, 20 min)	30°C - Decanter (3300 x g, HRT= 5 min)	30°C (4000 x g, 20 min)
		Disc (9000 x g, HRT= 1 min)	
Mixing	400 rpm during alkaline extraction; 100 rpm during acidification	3 kW in the first two compartments of the alkaline reactor; 0.55 kW in the acidification tank	400 rpm during alkaline extraction; 100 rpm during acidification

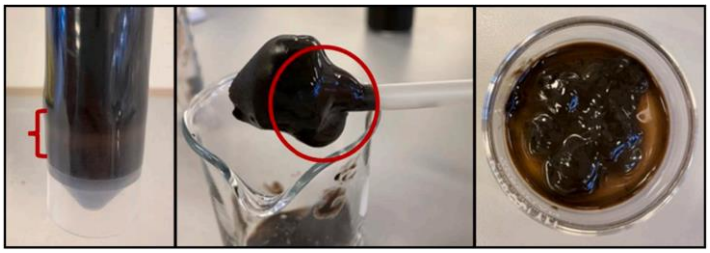

Figure 2: The appearance of an alkaline gel with the modified laboratory protocol (Bahgat et al., 2023).

3.2. METHOD DESCRIPTION

Collected excess thickened sludge sample (5% total solids [TS]) is subjected to a water bath to 80°C. 25% (w/v) KOH is added until pH 10 is reached (note: for optimal product quality this pH might be different and needs adaption, usually within the range of pH 9-11). The sample is mixed thoroughly for two hours. After letting it cool to room temperature, the mixture is centrifuged at 4,000 × g and 30 °C for 20 min. Afterwards, 30 wt% HCl is added to the alkaline supernatant, while mixing, until a final pH of 2.2 (note: for optimal product quality this pH might be different and needs adaption, usually within the range of pH 2-4). The acidified extract is then centrifuged at 4,000 × g and 30 °C for 20 min, and the gel-like EPS pellet collected.

3.3. PROCEDURE

The detailed lab extraction protocol can be seen in Appendix B.

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4. KAUMERA (AND SLUDGE) STORAGE

To mitigate the risk of microbiological decomposition of organic solids, it is advisable to store Kaumera (and sludge) at a temperature of 4°C, either in cold rooms or refrigerators, until the time of analysis (or extraction). The duration before degradation varies based on sample quality, exposure time to air before sealing, and pH of the sample. Low-pH Kaumera samples tend to have a longer storage life compared to high-pH samples (when pH is modified). This distinction arises from the fact that high-pH samples are more susceptible to hydrolysis and generally undergo degradation at a faster rate. Nevertheless, with proper storage, Kaumera generally maintains its quality for approximately four weeks before any visible signs of degradation, such as mold, become evident (M. Smit, personal communication, January 10, 2024). Adding 0.12% sodium benzoate (common food preservative) to Kaumera (approximately pH 2.2) can also significantly improve its shelf-life in terms of degradation. Alternatively, if the pH of Kaumera has been adjusted to pH 5-6, the addition of 0.4% potassium sorbate helps prevent mold formation. For extended storage, it is advisable to regularly inspect flow properties and measure volatile solids (VS), total solids (TS), and pH.

5. SAMPLE PREPARATION METHODS

The following are sample preparation methods commonly employed before characterization methods in the lab, depending on the research's end goal. This include washing or dialysis to reduce dissolved low molar mass compounds (e.g., salts) and drying if redissolution is required. On-site characterization methods (Chapter 7) typically do not involve these preparation steps.

Before implementing any sample preparation or characterization method, it is important to ensure that Kaumera is brought to room temperature. Similar to many other materials, biopolymers like Kaumera exhibit behavior that is influenced by temperature variations. This means that changes in temperature can affect their physical properties, including viscosity, solubility, and conductivity. Maintaining a consistent temperature throughout any procedure is ideal. Temperature used should be reported and temperature corrections made if necessary (e.g., reporting conductivity at 25°C) to facilitate meaningful result comparisons. In a similar manner, diluting the Kaumera also modifies the polymers' properties and behaviors and therefore should be well-documented.

Additionally, when Kaumera is stored for extended periods, it may experience segregation, in which solid components settle at the bottom of the storage container. Therefore, prior to initiating any method, the Kaumera sample should be thoroughly mixed to achieve a uniform and homogenous state. This ensures that the sample is consistent and representative for subsequent methods or analyses.

5.1. WASHING

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5.1.1. INTRODUCTION

Washing Kaumera gel is a post-processing step that can be utilized to reduce dissolved low molar mass compounds. The choice of the washing solution depends on the specific purpose and the substance(s) to minimize. Ideally, the pH, conductivity, and temperature of the washing solution should replicate those of the Kaumera supernatant (liquid phase), while adjusting solely the parameter related to the targeted removal. For instance, if the goal is to eliminate free-floating organic molecules, a solution that matches the Kaumera supernatant in terms of pH, conductivity, and temperature but lacks organic content should be selected. Note that although there may be differences in the concentration of specific species, if the pH, conductivity, and temperature are kept the same, there exists a lower risk of modifying the existing electrostatic interactions and changing the characteristics of the polymer. If the goal is to eliminate the dissolved excess ions (salts), a solution that matches the Kaumera supernatant in terms of pH and temperature is sufficient.

Below is an example of washing Kaumera with hydrochloric acid solution. With the HCl solution, it is possible to remove both the organic molecules and the counter ions present in the supernatant (unselective) and attain a far more homogeneous composition, as the whole liquid phase is exchanged with a well-defined HCl solution. It has been observed that washing Kaumera with hydrochloric acid reduces the EC roughly 80-90% and increases the VS approximately 4-5% (A. Raja, personal communication, September 5, 2023). This means that there is a substantial amount of non-volatile substances that can be easily removed. Furthermore, the practice of washing Kaumera has proven to be highly advantageous in increasing its rheological characteristics (A. Raja, personal communication, September 5, 2023). In case the Kaumera is used as a dry film e.g. as a coating, the presence of excess salts may cause defects, brittleness and loss of strength.

5.1.2. METHOD DESCRIPTION

This procedure involves diluting the Kaumera sample by a factor of two using a hydrochloric acid (HCl) solution in Milli-Q water with an equal pH to the Kaumera sample. Following this, a centrifugation step is implemented to separate the pellet (containing the polymer) from the supernatant, which is then discarded. This process effectively eliminates the free ions present in the supernatant by dilution. These steps are iterated while continuously monitoring both the pH and conductivity until a consistently low conductivity value is attained.

5.1.3. PROCEDURE

The step-by-step instructions for implementing this washing method are outlined in Appendix C.

5.2. DIALYSIS

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5.2.1. INTRODUCTION

Dialysis is a separation method employed to selectively remove undesired small compounds from larger macromolecules dissolved in a solution. This process relies on the passive diffusion of substances through a semi-permeable membrane (De Castro, 2008). To perform dialysis, a sample solution and a buffer solution or dialysate are positioned on opposite sides of the membrane.

The molecules in the sample that are larger than the pores of the membrane are trapped on the sample side, while smaller molecules freely pass through the membrane, resulting in a decrease in their concentration within the sample. By periodically changing the dialysate buffer, the small molecules that have diffused out of the sample are removed, allowing more contaminants to migrate into the dialysate. In this manner, the concentration of undesirable small substances within the sample can be reduced to acceptable or negligible levels.

Depending on the purpose and the desired substance to remove, the dialysate and the pore size-range of the dialysis membrane, often referred to as the molecular weight-cutoff (MWCO), are selected. The MWCO is typically expressed in Da or kDa. Molecules that have a smaller size than the MWCO selected will be potentially removed from the sample.

5.2.2. METHOD DESCRIPTION

The method described here is a standard dialysis procedure used at TU Delft for the purification of EPS samples. The procedure aims to selectively remove dissolved low molar mass (<3.5 kDa) compounds (e.g., salts) ensuring the retention of the polymeric substances. For most characterization methods (i.e., BCA assay, phenol-sulfuric acid method, HPAEC-PAD, SEC, FTIR) it is recommended to remove salts as to not interfere with the analysis. Furthermore, removing salts aids in ensuring accurate comparisons, particularly when samples exhibit significant variations in salt content.

In this procedure, the sample is placed in a dialysis membrane with a 3.5 kDa MWCO. It is then positioned in a beaker filled with Milli-Q water, making sure it floats. Employing a magnetic stirrer, it is mixed thoroughly. The Milli-Q water is subsequently changed three to four times, with each change lasting approximately 4 hours.

5.2.3. PROCEDURE

The procedure is outlined in detail in Appendix D.

5.2.4. ADDITIONAL CONSIDERATIONS

This method has the potential to remove molecules with a size smaller than 3.5 kDa, risking the removal of monosaccharides and amino acids dissolved in the liquid phase of Kaumera. Therefore, this should be considered when the intention is to later quantify these substances.

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5.3. DRYING: FREEZE DRYING

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5.3.1. INTRODUCTION

For specific characterization methods, it is beneficial to work with dried Kaumera, especially when redissolving is necessary and when total solids content vary depending on the Kaumera. Various drying methods, including freeze drying, spray drying, or air drying, can be employed. The choice of drying method requires careful consideration, as each method can impact properties and behaviors differently (S. Picken, personal communication, November 24, 2023). Additionally, it is essential to ensure that the drying temperature remains below 60°C to minimize the risk of altering the EPS structure. Among the drying techniques, freeze drying is considered less damaging.

Freeze-drying or lyophilization is a very common technique used to remove moisture from a diverse range of substances. In this process, water in the form of ice under low pressure is removed from the material by sublimation. In other words, ice water is transformed to vapor, omitting the liquid state. Freeze drying can remove about 98-99% of the moisture content and offers several advantages: (1) reduces the risk of thermal degradation as it operates at low temperature, (2) good shelf-life stability, as it reduces the potential for microbial growth, and (3) allows for easy rehydration if the product needs to be redissolved (Arufe et al., 2020).

5.3.2. METHOD DESCRIPTION

The sample is enclosed in a plastic container and sealed with parafilm, with perforations made in the parafilm to facilitate the release of water vapor. Subsequently, the sample is frozen entirely by placing it in a freezer at -80°C. Following this, the frozen sample is transferred to the freeze-drier. The duration of the process varies based on the water content of the sample and its surface-to-volume ratio.

5.3.3. PROCEDURE

The detailed procedure can be found in Appendix E.

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6. LAB CHARACTERIZATION METHODS

6.1. VOLATILE SOLIDS (VS) AND TOTAL SOLIDS (TS)

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6.1.1. DEFINITIONS

As defined by the American Public Health Association (APHA, 1999):

- Total solids (TS): material residue left in the vessel after evaporation and succeeding drying in an oven at 105°C.
- Fixed solids or ash (A): residue of total solids after heating to dryness in a muffle furnace at 550°C.
- Volatile solids (VS): The weight that is lost on ignition in a muffle furnace at 550°C.

6.1.2. INTRODUCTION

Measuring total solids (TS) and volatile solids (VS) is a well-established practice in wastewater treatment. In the case of Kaumera, these measurements are fundamental as characterization parameters. Variations in concentration can lead to significantly distinct properties and behaviors among samples. Therefore, it is necessary to report both VS and TS when conducting characterization tests. Moreover, in the Kaumera production units, the assessment of VS and TS enables the monitoring of product quality. Adjustments to the centrifuge settings during the extraction procedure can also be made to achieve the desired TS level. Typically, Kaumera samples can have a total solids content ranging from 5-12% and a volatile content of approximately 70% of the TS (STOWA, 2019).

The method used to measure TS and VS is based on the "Standard Methods for the Examination of Water and Wastewater" (1999) by the American Public Health Association (APHA). It is worth noting that this method does not provide a precise differentiation between organic and inorganic matter. This is because the loss on ignition includes not only organic matter but also losses from the decomposition or volatilization of certain mineral salts.

6.1.3. METHOD DESCRIPTION

Kaumera samples are subjected to overnight drying at 105°C to obtain the total solids (TS). The samples are taken out and cooled in the desiccator for 15 minutes. Subsequently, they are subjected to a two-hour combustion process at 550°C. Again, the samples are placed in the desiccator for 15 minutes, and the volatile solids (VS) and ash (A) determined. Measurements are performed on triplicates.

6.1.4. PROCEDURE

The detailed method for measuring VS and TS for Kaumera samples can be seen in Appendix F. The method described here is based on the procedure outlined in "Standard Methods for the Examination of Water and Wastewater" (1999) by the American Public Health Association (APHA).

6.1.5. SOURCES OF ERROR

- Always pre-ash the evaporating dish prior to taking measurements. If volatile solids are to be measured, ignite clean evaporating dish at 550°C for one hour in a muffle furnace. If only total solids are to be measured, heat the clean dish to 105°C for one hour. This is to ensure residual material from the production of the dishes are completely removed and is not taken into account as part of the ash weight.

- Make all weighing quickly as wet samples lose weight by evaporation. After drying or ignition, residues are also hygroscopic and can rapidly absorb moisture from the air.
- When a portion of the sample adheres to the surface, it is important to take this into account when assessing and documenting the results. In certain cases, samples may form a crust during drying, which can impede water evaporation.
- Reduce the frequency of opening the desiccator as it allows humid air to enter.
- Residues dried at 105°C can retain crystallized water and mechanically occluded water.
- It is important to note that due to the fire-retardant characteristics of Kaumera, there exists the risk that complete combustion may not have occurred after the established two hours. For more precise and accurate results, it is an option to extend the samples' exposure in the oven until the weight change is less than 4%.
- Ceramic crucibles are preferred sample holders over aluminum cups, as the quality of the aluminum cup can differ per manufacturer and influence the measurement.

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6.2. PROTEINS

Kaumera's primary component is proteins, comprising 50-70% of the organic matter (STOWA, 2019). Thus, quantifying proteins is needed for a comprehensive understanding of each individual polymer and for comparison among samples. The measurement of protein content also holds potential significance in agricultural contexts, particularly due to the association of specific amino acids, such as tryptophan, with biostimulation (Chiaiese et al., 2018).

The protein content of Kaumera can be influenced by several factors, including the choice of precipitation acid (S. Picken, personal communication, May 6, 2023) and the SRT in Nereda reactors (Zhang et al., 2019; Mahendran et al., 2015). This indicates there may exist potential opportunities for process adjustments to achieve the desired protein content.

6.2.1. BICINCHONINIC ACID (BCA) ASSAY

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6.2.1.1. INTRODUCTION

The BCA assay is a widely employed colorimetric method in EPS research for total protein quantification. It is conveniently accessible as a commercial kit (e.g., Pierce™ BCA Protein Assay Kits, Thermo Fisher Scientific™), making it user-friendly. This method operates based on a similar principle as the Lowry Method, but it offers a simplified approach and has demonstrated more tolerance to many substances known to interfere with the Lowry Method (Brown et al., 1989). To establish standard curves, bovine serum albumin (BSA) is typically used, and measurements are conducted in a 96-well plate, with absorbance readings taken at 562 nm.

This method consists of the reduction of Cu^{2+} to Cu^{+1} by protein in an alkaline medium (the biuret reaction). Using the reagent bicinchoninic acid (BCA), it is possible to detect the cuprous cation (Cu^{+1}) via spectrophotometry. The purple-colored reaction product of this test is formed by the chelation of two molecules of BCA with one cuprous ion. Furthermore, the formation of color is attributed to specific factors within protein macromolecular structure, including the number of peptide bonds and the presence of four specific amino acids: cysteine, cystine, tryptophan, and tyrosine.

Like many other colorimetric methods, the BCA assay is straightforward, rapid, and demands minimal equipment. Nevertheless, it does come with specific limitations related to the absence of appropriate standard compounds and potential cross-interferences (for more information, refer to Section 6.2.1.4.). Consequently, this method should primarily serve to provide a general understanding of the EPS composition (Felz et al. in 2019). If a higher level of precision is required, an alternative approach is available for measuring amino acids using GC-MS (Section 6.2.2.).

6.2.1.2. METHOD DESCRIPTION

Each standard of BSA (working range = 0-2,000 ug/ml; triplicates) and Kaumera sample (triplicates) is placed in a microplate well. BCA is added to each well and the plate is mixed thoroughly on a plate shaker for 30 seconds. The plate is covered and incubated at 37°C for 30 minutes. Afterwards, the plate is cooled to room temperature and the absorbance is measured at 562 nm on a plate reader. The average 562 nm absorbance measurement of the blank standard replicates is subtracted from each individual standard and Kaumera sample. A standard curve (average blank–corrected 562 nm measurement for each BSA standard vs. concentration in µg/ml) is constructed to determine the protein concentration of each Kaumera sample. In principal, the absorbance for the standards should always be the same. However, it is recommended to create a new calibration line each time measurements are taken, especially if the instrumentation changes. This practice ensures precision and accuracy, providing assurance that both the

analyst and the instrument are performing as expected. Results are often expressed in terms of volatile solids (% of volatile solids or g/g VS) to exclude the inorganic mass of the solvent.

6.2.1.3. PROCEDURE

Before performing the BCA assay, it is necessary to subject the Kaumera samples to a specific sample preparation method, outlined in detail in Appendix G. The protocol used at TU Delft is the "Pierce™ BCA Protein Assay Kit (Catalog Numbers 23225 or 23227)". Thermo Fisher. https://www.thermofisher.com/order/catalog/product/es/es/23225. Published January 30, 2020.

6.2.1.4. SOURCES OF ERROR

- There is a possibility that freely moving amino acids are eliminated during the dialysis process, which is part of the sample preparation method. Nevertheless, this is inconsequential if the focus of the study is on characterizing the polymer.
- If the Kaumera is not completely dissolved, meaning it is precipitating, it would lead to an improper analysis.
- As mentioned before, peptide bonds and specific amino acids are responsible for the color reaction observed in this assay. Since Kaumera contains proteins with a different composition to that of the used standard, this can result in an over- or under-estimation of the protein content in the sample (Felz et al., 2019).
- Cross-interferences can arise due to the presence of other constituents in Kaumera, such as humic acid, gallic acid, and glucosamine (Felz et al., 2019). Small interferences have also been observed by glucose and galacturonic acid.
- When constructing the calibration line for the standards, consider only those data points that demonstrate a linear trend. At higher standard concentrations, the line begins to curve, introducing potential inaccuracies.

6.2.1.5. ADDITIONAL CONSIDERATIONS

The BCA method doesn't provide a true endpoint; instead, the color development continues over time. However, after incubation, the rate of this ongoing color development is gradual enough to facilitate the analysis of a large batch of samples simultaneously.

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6.2.2. GAS CHROMATOGRAPHY MASS SPECTROMETRY (GC-MS)

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6.2.2.1. DEFINITIONS

- Retention time: time between injection and elution of a sample component.
- Mass-to-charge ratio (m/z): represents the mass given to a particle compared to the electrostatic charge it carries.
- Silylation: derivatization procedure for sample analysis that involves the introduction of "silicon groups" (also known as "silyl groups") into molecules (refer to Figure 3). This is achieved by esterifying the metabolite using N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA), with the addition of N-Methyl-N(tert-Butyldimethylsilyl Trifluoroacetamide) (TBDMCS) as a catalyst. During this process, the acidic protons in carboxyl, hydroxyl, primary amines, secondary amines, and thiol groups are replaced with tert-butyldimethylsilyl (TBDMS) groups. These TBDMS groups provide enhanced volatility and thermal stability to the metabolite.

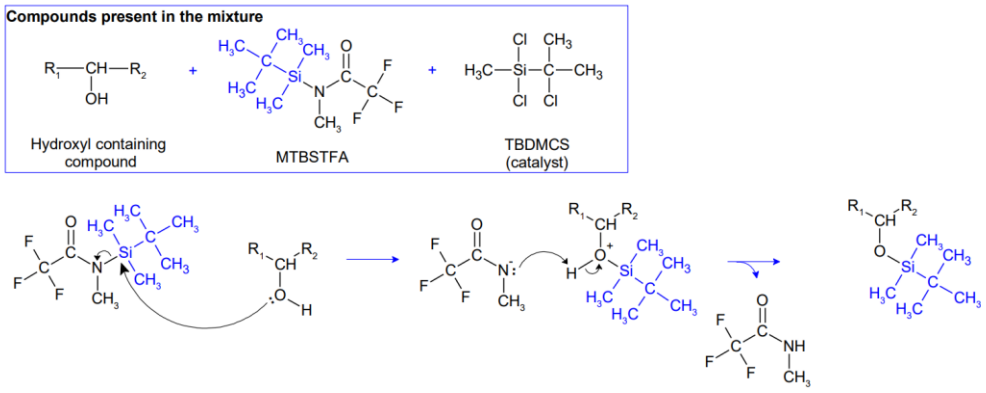

Figure 3: Derivatization process of silylation used for sample preparation for GC-MS.

6.2.2.2. INTRODUCTION

Gas chromatography – mass spectrometry (GC-MS) is an instrumental method employed for the separation, quantification, and analysis of naturally volatile organic compounds (Surat, 2019). It consists of two different analytical techniques: gas chromatography (GC) and mass spectrometry (MS). Gas chromatography (GC) is a method utilized to separate substances, including amino acids, within a sample mixture. Subsequently, it facilitates the detection of these compounds to establish whether they are present, absent, or to quantify their concentrations. GC detectors typically provide limited information, usually in a two-dimensional format, containing the retention time on the analytical column and the detector response (Turner, 2022). Quantification relies on comparing the retention time of peaks in the sample with those derived from standards of known concentrations analyzed using the same method. Nevertheless, GC alone is insufficient for identifying unknown substances, which is where coupling with mass spectrometry (MS) proves highly effective. Mass spectrometry (MS) is an analytical method that measures the mass-to-charge ratio (m/z) of charged particles, enabling the determination of molecular weight and elemental composition while also unraveling the chemical structures of molecules. Therefore, in the case of GC-MS, the data generated is three-dimensional, encompassing mass spectra useful for confirming identities or identifying unknown compounds, in addition to chromatograms that serve the purpose of both qualitative and quantitative analysis.

While the BCA assay (Section 6.2.1.) is valuable for rapid and convenient determination of total protein content, GC-MS offers a more detailed and specific analysis when the focus is on understanding the composition of individual

amino acids in a sample while offering high sensitivity and robustness for analyzing complex samples. The choice between these methods depends on the specific research goals and the information required.

6.2.2.3. METHOD DESCRIPTION

To enhance the volatility, stability, and detectability of samples, a derivatization process known as silylation is conducted on hydrolyzed (freeze) dried Kaumera samples. After the sample has been derivatized and centrifuged, it is introduced into the GC system. Within the heated GC inlet, the liquid sample transforms into vapor (note: only the compounds that are volatilized will pass through the column, while the non-volatilized substances will remain at the inlet). The compounds are then separated based on their volatility and interactions with a coated column (stationary phase) and a carrier gas, typically helium (mobile phase). This separation process helps resolve individual amino acids from the mixture. Subsequently, these compounds elute from the column and enter the mass spectrometer.

Inside the mass spectrometer, the neutral molecules undergo a process of ionization, most commonly using electron ionization (EI), which results in the creation of charged particles. However, due to the high energy applied during ionization, the molecules may become unstable and break into smaller fragments. Then, the mass analyzer sorts these ions based on the m/z value of the fragments. Each derivative of an amino acid produces a distinctive mass spectrum, serving as a unique identifier. In other words, the m/z ratio serves as a fingerprint for each amino acid. Finally, the ions are detected by the ion detector, enabling the generation of a chromatogram and a mass spectrum for each data point.

6.2.2.4. PROCEDURE

The detailed procedure followed at TU Delft can be seen in Appendix H.

6.2.2.5. DATA ANALYSIS

The data obtained from GC-MS is three-dimensional, as illustrated in Figure 4. The x-axis represents the retention time and the y-axis shows the response or intensity measured by the ion detector, which is a measure of the number of ions at the point of retention. Furthermore, the z-axis corresponds to the m/z values of the ions. Each peak in the chromatogram corresponds to an amino acid, and thus each peak corresponds to an individual mass spectrum and can typically be accessed separately in the software, often in a separate window.

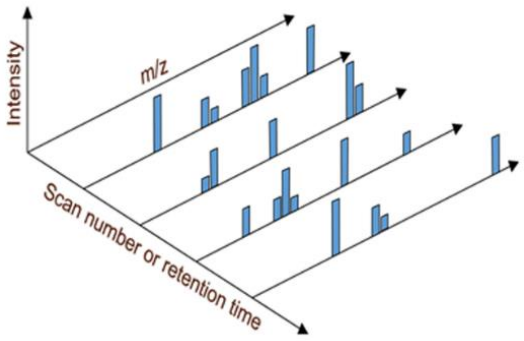

Figure 4: Three-dimensional data output for GC-MS (Turner, 2022).

In gas chromatography (GC), the retention times are used to identify the target analytes, while the areas under the peaks are commonly employed for quantification. This is because the peak area is directly proportional to the analyte concentration. To determine the monosaccharide concentrations of an unknown sample, known standards with varying concentrations are typically used. However, it is highly recommended to use an internal standard to enhance

the precision of the analysis and account for systematic errors. If this is applied, results are not calculated by the peak area of the target analyte but by using the peak area ratio:

Peak area ratio = "Peak area of analyte" / "Peak area of internal standard"

This approach allows for the adjustment of variations in the sampling and analysis process. Since the internal standard is uniformly added to all standards and samples at an identical concentration, the internal standard responds to the same conditions as the target analyte, making it possible to correct for variations and minimize their impact on the results.

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6.3. CARBOHYDRATES

Following proteins, polysaccharides constitute the second most abundant component in Kaumera, making up approximately 25% of the total organic matter (STOWA, 2019). Consequently, it is necessary to quantify carbohydrate content to achieve a thorough understanding of each individual polymer. Additionally, carbohydrates may be linked with the gel-like or adhesive properties of Kaumera. It has been observed that sugars play a crucial role in Kaumera's gel formation, with higher sugar content contributing to better shape retention (De Roos, 2022). Studies also suggest that polysaccharides enhance EPS adhesion strength, while proteins exhibit lesser adherence effects (Harimawan & Ting, 2016; Herald & Zottola, 1989; Mai et al., 1993). Various factors, such as microbial species, carbon substrate, nutrients (N, P), extraction method, and AGS operation process, can affect EPS carbohydrate content (Klai et al., 2017).

6.3.1. PHENOL-SULFURIC ACID METHOD

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6.3.1.1. INTRODUCTION

The phenol-sulfuric acid method established by Dubois et al. (1956) is one of the most common colorimetric assays used to determine the total carbohydrates in a sample. The concentrated sulfuric acid breaks down any polysaccharides, oligosaccharides, and disaccharides to monosaccharides (Nielsen, 2009). Pentoses (5-carbon compounds) are dehydrated to furfural, while hexoses (6-carbon compounds) to hydroxymethyl furfural. The phenol is added to react with these compounds and produce a yellow-gold color. Although the method detects all types of carbohydrates, the absorptivity of the different carbohydrates varies, and thus the result is typically expressed in terms of one carbohydrate. Since glucose is the most commonly applied standard in EPS research, it has also been used to create the standard lines for Kaumera samples in previous studies (Felz et al., 2019).

Like many other colorimetric methods, the phenol-sulfuric acid method is straightforward, rapid, and demands minimal equipment. Nevertheless, it does come with specific limitations related to the absence of appropriate standard compounds and potential cross-interferences (for more information, refer to Section 6.3.1.4.). Consequently, this method should primarily serve to provide a general understanding of the EPS composition (Felz et al. in 2019). If a higher level of precision is required, an alternative approach is available for separating and quantifying monosaccharides using HPAEC-PAD (Section 6.3.2.).

6.3.1.2. METHOD DESCRIPTION

Various concentrations of standard glucose (working range = 0 to 200 mg/L; triplicates) and dissolved Kaumera samples (triplicates) are placed in glass borosilicate tubes. A solution of 95-97% sulfuric acid is introduced to generate furan derivatives. Afterward, phenol is added to condense these furan derivatives, resulting in the formation of stable yellow-gold compounds. These solutions are subsequently pipetted into test tubes and subjected to measurement using a spectrophotometer.

Before proceeding, it is advisable to perform a spectrum scan to verify that the absorbance maxima for glucose falls on, or is very close to, 490 nm. Reporting absorbances at 490 nm will simplify the comparison process.

To calculate the glucose equivalent within each sample, the average absorbance measurement at 490 nm for the blank standard replicates is subtracted from the absorbance of each individual standard and Kaumera sample. A calibration line is constructed using the standards, and then the absorbance intensity of the Kaumera samples is compared to that of the glucose standard, for which the concentrations are known. In principal, the absorbance for the standards should always be the same. However, it is recommended to create a new calibration line each time

measurements are taken, especially if the instrumentation changes. This practice ensures precision and accuracy, providing assurance that both the analyst and the instrument are performing as expected. Results are often expressed in terms of volatile solids (% of volatile solids or g/g VS) to exclude the inorganic mass of the solvent.

6.3.1.3. PROCEDURE

The detailed procedure used at TU Delft can be seen in Appendix I.

6.3.1.4. SOURCES OF ERROR

- There is a possibility that freely moving glucose and other sugars are eliminated during the dialysis process, which is part of the sample preparation method. Nevertheless, this is inconsequential if the focus of the study is on characterizing the polymer.
- If the Kaumera is not completely dissolved, meaning it is precipitating, it would lead to an improper analysis.
- Utilizing glucose as a standard oversimplifies the varied carbohydrate composition found in Kaumera. Previously, a mixture of sugars was employed as a standard to provide a more accurate representation of EPS (Felz et al., 2019). Nevertheless, this approach requires prior knowledge of which sugars are present through HPAEC-PAD (Section 6.3.2.) analysis to create the sugar mixture. Hence, expressing concentrations in terms of glucose equivalents presents a simpler and faster alternative.
- There exist interferences by the presence of other components present in Kaumera such as uronic acids (e.g., galacturonic acid) and humic substances (Felz et al., 2019). This can lead to an overestimation of the measured saccharide content.

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6.3.2. HIGH-PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY WITH PULSED AMPEROMETRIC DETECTION (HPAEC-PAD)

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6.3.2.1. INTRODUCTION

High-Performance Anion Exchange Chromatography (HPAEC) performed under alkaline conditions and combined with Pulsed Amperometric Detection (PAD) is a highly valuable method for carbohydrate analysis. This technique allows for the separation of simple sugars, oligosaccharides, and polysaccharides, as well as accurate quantification with exceptional resolution in a single analytical run (Corradini et al., 2012).

Many carbohydrates are weak acids with pKa values in the range of 12-14 (Corradini et al., 2012). Consequently, at high pH levels, their hydroxyl groups undergo partial or complete transformation into oxyanions. This transformation allows this class of compounds to be selectively eluted as anions using high-performance anion-exchange chromatography. Typically, a quaternary-ammonium-bonded pellicular anion-exchange column is used for carbohydrate separation in alkaline conditions.

Unlike the phenol-sulfuric acid method, HPAEC-PAD stands out by allowing for the specific identification and quantification of individual monosaccharides in a sample. In contrast, the phenol-sulfuric acid method provides a total carbohydrate measurement without distinguishing between specific sugar types. In addition, HPAEC-PAD offers advantages in terms of quantitative accuracy, sensitivity, resolution, and suitability for complex samples. The choice between these methods depends on the specific research goals and the desired level of detailed information.

6.3.2.2. METHOD DESCRIPTION

For Kaumera, it is first necessary to undergo a hydrolysis step in order to break down the linkages that connect the sugar polymers to obtain monomers. It is important to note that the critical factors influencing this process include both the duration and conditions of hydrolysis. Sugar monomer standards are prepared in order to create calibration curves. Apart from having the normal samples diluted in Milli-Q water, it is also necessary to prepare spiked samples which mix the sample and the standards due to possible salt interference.

The samples are injected into the HPAEC-PAD system with an alkaline eluent solution as the carrier (mobile phase). Depending on the type of sugar to target, a specific eluent solution is chosen. The samples travel through an ion exchange column, which contains resin beads with positively charged groups (quaternary ammonium) that attract and retain negatively charged ions from the sample. As the sample flows through the column, the ions are separated based on their charge and affinity for the resin. The more strongly charged ions are retained longer (longer retention time), while the less strongly charged ions pass through the column faster (shorter retention time). Next, the ions pass through PAD, a highly sensitive electronic detection method. PAD works by applying voltage pulses to a working electrode. As the ions elute, they reach the electrode and undergo electrochemical reactions. These reactions generate electrical currents that are directly proportional to the concentration of the ions.

6.3.2.3. PROCEDURE

The detailed procedure applied at TU Delft can be seen in Appendix J.

6.3.2.4. DATA ANALYSIS

After completing the HPAEC-PAD analysis, a chromatogram is generated. The y-axis displays electrical charge measured in nanoCoulombs (nC), while the x-axis represents retention or elution time (see Figure 5). By overlaying the chromatograms of the spiked and normal samples, the sugar peaks can be qualitatively visualized. Each distinct peak within the chromatogram corresponds to a specific sugar, as each sugar possesses a unique and identifiable retention time. It is important to highlight that it is most probable that the expected sugar peaks are shifted. However, the order in which the sugar peaks appear is always the same, as the order of increasing retention is correlated with decreasing pKa values (e.g., first fucose, then galactosamine, followed by glucosamine, etc.).

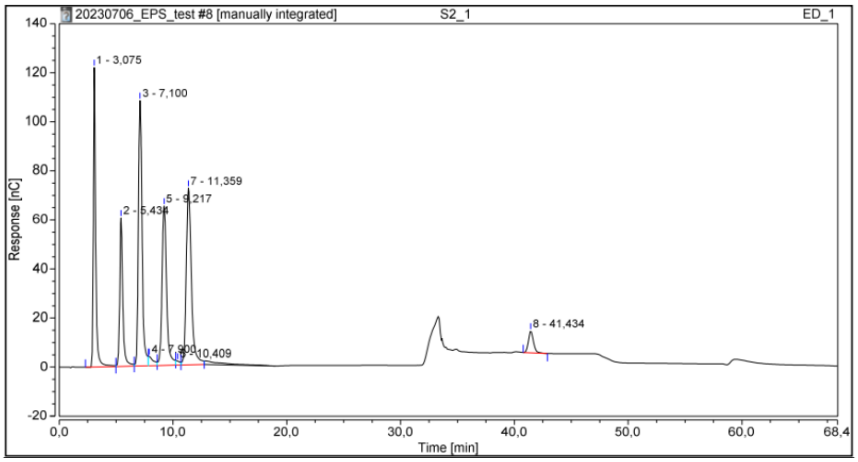

Figure 5: Example of chromatogram from HPAEC-PAD for a Kaumera sample.

Each peak area on the chromatogram corresponds directly to the quantity of a specific sugar present in the sample. This enables the construction of calibration curves, where peak area (nC.min) is plotted against concentration. As a result, it is possible to quantify a specific sugar within the sample by comparing the peak area of the sample to the peak areas of known standards of the same sugar at known concentrations.

Furthermore, the peak shape can provide information about the purity and efficiency of separation. A well-defined, symmetrical peak suggests a clean separation, while a broad or asymmetric peak indicate issues with resolution or column performance.

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6.4. PROTEINS AND CARBOHYDRATES: INDIRECT MEASUREMENTS

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6.4.1. DEFINITIONS

As defined by Kleerebezem and van Loosdrecht (2006):

- Chemical oxygen demand (COD): quantity of oxygen equivalents needed to completely oxidize all organic carbon to carbon dioxide, often expressed in $gO_2.kg^{-1}$.
- Organic dry matter content (ODM): equivalent to volatile solids (VS), refers to the percentage of dry matter in a substance composed of organic substances, such as carbon-containing compounds after removing water and inorganic compounds, typically expressed in $g_{ODM}.kg^{-1}$.
- Total nitrogen (TN): sum of nitrate nitrogen (NO_3-N), nitrite nitrogen (NO_2-N), amine nitrogen (NH_4-N), and organic bounded nitrogen ($g_N.kg^{-1}$).
- Total Kjeldahl nitrogen (TKN): sum of the organic bounded nitrogen groups and the ammonium nitrogen (NH_4^+) ($g_N.kg^{-1}$).
- Organic nitrogen (N_{org}): estimated by subtracting the concentration of ammonium (NH_4^{+1}) from the TKN, commonly expressed as $g_N.kg^{-1}$.

6.4.2. INTRODUCTION

This section outlines an indirect method for measuring the total protein and carbohydrate content in Kaumera. This approach serves as an alternative to the methods described in Section 6.2.1. and Section 6.3.1. or can be employed for result validation. However, it is important to note that these calculations offer an approximate estimation as assumptions and generalizations are made.

By determining the elemental composition of biomass with the formula $C_cH_hO_oN_n$ it is possible to estimate its composition in relation to the primary building blocks, which include proteins, carbohydrates, and lipids. The following methodology was obtained from "Chapter 14 - Biochemical Conversion: Anaerobic Digestion" (Kleerebezem, 2014) from "Biomass as a Sustainable Energy Source for the Future: Fundamentals of Conversion Processes" (De Jong & Van Ommen, 2014).

To start, the COD value of organic matter can be identified via the combustion equation, which can be expressed in general terms as:

$$C_cH_hO_oN_n + xO_2 \rightarrow yH_2O + zCO_2 + wNH_3 \qquad (1)$$

Here, the coefficient x=(4+h-2o-3n)/4 denotes the number of oxygen molecules needed to combust one mole of organic carbon (c=1). By utilizing the molar weights of the various compounds, the COD/ODM ratio is defined:

$$\frac{COD}{ODM} = \frac{32(4+h-2o-3n)/4}{(12+h+16o+14n)} \qquad (2)$$

From the organic nitrogen measurement, it is possible to derive an equation for the coefficient n:

$$n = \frac{N_{org}(12+h+16o+14n)}{14ODM} \qquad (3)$$

Furthermore, since carbon is always tetravalent and assuming C–H, C = O, and C–NH_2 as carbon bonds, the following equation is derived:

$$4 = h + 2o - n \qquad (4)$$

By solving these three equations, the elemental composition of the organic carbon compound with c=1 can be obtained:

$$h = \frac{308COD + 704N_{org}}{49COD - 64N_{org} + 112ODM} \qquad (5)$$

$$o = \frac{224ODM - 56COD - 304N_{org}}{49COD - 64N_{org} + 112ODM} \qquad (6)$$

$$n = \frac{352N_{org}}{49COD - 64N_{org} + 112ODM} \qquad (7)$$

Subsequently, the carbon mole fraction ($mol_x.mol_C^{-1}$) of proteins (η_{PR}), lipids (η_{LIP}), and carbohydrates (η_{CHO}) can be calculated.

The protein fraction (η_{PR}) calculation is straightforward as protein is the only nitrogen-containing compound assumed. Thus, it is calculated from the N-content of the biomass (n) and the N-content of the standard amino acid composition of the protein chosen (N_{PR}). In this case, N_{PR} = 0.26 $mol_n.mol_C^{-1}$ is used (see Table 2).

$$\eta_{PR} = \frac{n}{N_{PR}} \qquad (8)$$

The fraction for lipids (η_{LIP}) can be determined by assessing the distinct oxidation states of lipids (γ_{LIP}) in comparison to the other organic substrates defined. In this case, γ_{LIP}= 5.68 $mol_e.mol_C^{-1}$ is assumed (see Table 2).

$$\eta_{LIP} = \frac{h - 2o - 3n}{\gamma_{LIP} - 4} \qquad (9)$$

Finally, from the total balance it is possible to calculate the remaining fraction of carbohydrates:

$$\eta_{CHO} = 1 - \eta_{PR} - \eta_{LIP} \qquad (10)$$

Table 2: Principal compounds considered as general organic feedstock constituents (Kleerebezem, 2014).

Compounds[a]	Abbr.	Chemical Composition	Oxidation State[b] (γ)($mol_e.mol_C^{-1}$)	N-content ($mol_N.mol_C^{-1}$)
Proteins	PR	$C_1H_{2.52}O_{0.87}N_{0.26}$	4.0	0.26
Carbohydrates	CHO	$C_1H_2O_1$	4.0	0.0
Lipids	LIP	$C_1H_{2.85}O_{0.575}$	5.7	0.0

Note: The elemental composition of proteins relies on the generalized amino acid composition proposed by Batstone et al. (2002). Carbohydrates are determined based on the composition of glucose ($C_6H_{12}O_6$).

[a]For the chosen model lipid, it consists of one glycol molecule and three n-palmitic acid side chains, resulting in an elemental composition of $C_{51}H_{98}O_6$.

[b]The substrate's oxidation state is defined as the number of electrons liberated per C-mol substrate upon full oxidation to carbon dioxide, aligning with the previously described COD definition (Heijnen and Kleerebezem, 2010).

6.4.3. METHOD DESCRIPTION

By measuring COD, total nitrogen, ammonium concentration, and ODM it is possible to obtain an estimate of the proportion of total carbohydrates, proteins, and lipids in a sample. COD is measured with a commercial Hach Lange COD kit (LCK 014). Total nitrogen can be measured with a commercial Hach Lange Kit (LCK 138 Laton Total Nitrogen) or with an element analyzer. The total organic nitrogen is obtained by subtracting the ammonium concentration from the total nitrogen. Note that in Kaumera, nitrate and nitrite are negligible (Bahgat et al., 2023). ODM is obtained by conducting the procedure described in Section 6.1. With these measurements, equations (5), (6), and (7) are applied in order to obtain the biomass composition $C_cH_hO_oN_n$. Afterwards, the fractions for proteins, carbohydrates, and lipids can be calculated with equations (8), (9), and (10).

6.4.4. PROCEDURE

A detailed description of the procedure can be found in Appendix K.

6.4.5. ADDITIONAL CONSIDERATIONS

It is possible to replace COD measurements with Total Organic Carbon (TOC). To do so, it is necessary to initially establish a correlation between the two (e.g., COD = 49.2 + 3.00*TOC) for each extraction location (Dubber & Gray, 2010). Afterward, only TOC measurements will be needed for further analysis.

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6.5. FRACTIONATION: SIZE EXCLUSION CHROMATOGRAPHY (SEC)

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6.5.1. INTRODUCTION

EPS are composed of a wide range of biopolymers, including proteins, polysaccharides, nucleic acids, and lipids. In order to better analyze their structure, function, and interactions, it is useful to fractionate EPS due to the complexity of the biological matrices. Size Exclusion Chromatography (SEC) is the most applied separation technique available for polymers (Deb et al., 2019). SEC allows for the separation of these components based on their molecular weights. It is also a quantitative tool that can be used to determine the relative abundance between the different components. Once EPS components are separated via SEC, they can also be collected in individual fractions and analyzed via FTIR, GC-MS, HPAEC-PAD, among others.

6.5.2. DEFINITIONS

- Geometric column volume (V_c): physical volume of column (see Figure 6a).
- Void volume (V_o): equivalent to the elution volume of a large molecule, like Blue Dextran, which is excluded from all pores.
- Volume of solvent retained (V_s): volume of solvent retained in the pores, which is approximated to V_c - V_o.
- Elution volume (V_e): volume of solvent between injection and elution (see figure 6b).

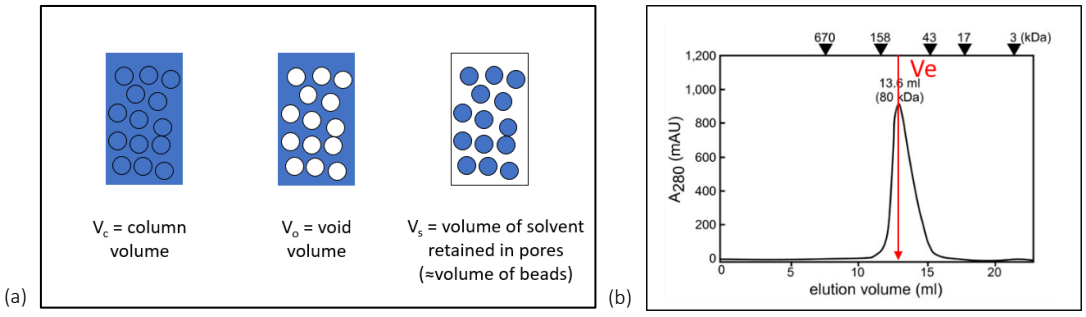

Figure 6: (a) Graphic representation of V_c, V_o, V_s, (b) V_e.

- Partition coefficient (K_{av}): coefficient that describes the proportion of pores available to the molecule. For a molecule that can partially enter the pores:

$$V_e = V_o + K_{av}(V_c - V_o) \qquad (11)$$

- When the molecule is totally "excluded" due to its large size: $K_{AV} = 0$ and thus $V_e = V_o$.
- When the molecule is totally "included" due to its small size: $K_{AV} = 1$ and thus $V_e = V_c$.

Thus, K_{av} can be calculated as follows:

$$K_{av} = \frac{V_e - V_o}{(V_c - V_o)} \qquad (12)$$

6.5.3. METHOD DESCRIPTION

SEC is a liquid chromatography method that accomplishes the separation of molecules based on their size when they are in solution. The size-based separation takes place within a column that contains a gel (stationary phase). The gel consists of spherical beads that contain different size pores. To conduct SEC, the sample is dialyzed (Section 5.2.), freeze dried (Section 5.3.), and then dissolved in a buffer solvent (mobile phase) and introduced into the column. Large molecules in the sample cannot enter through the pores and are eluted faster, whereas smaller molecules can diffuse into the pores and thus their flow through the column is delayed according to size. After molecules elute, they pass through the detector in which they absorb UV light, which result in the absorbance signal used to generate a chromatogram. Furthermore, with the use of a calibration curve (K_{av} values vs. logarithm of molecular weights), it is possible to obtain the molecular weight range of each fraction once their K_{av} values are determined.

6.5.4. PROCEDURE

The detailed sample preparation, procedure, and calibration used at TU Delft can be seen in Appendix L.

6.5.5. DATA ANALYSIS

In a SEC chromatograph, the y-axis represents the absorbance intensity at the given wavelength, and the x-axis can either be elution volume or time. The x-axis in a volume-based chromatograph represents the volume of eluent (mobile phase) that has passed through the column. On the other hand, in a time-based chromatograph, the x-axis demonstrates how long it takes for each component to travel through the system, as depicted in Figure 7. Each peak in the chromatograph represents a specific compound or analyte in the mixture. Therefore, fractions are commonly separated according to peak width.

In addition, the height of the peak is directly proportional to the concentration of the analyte and thus the area (mAU*ml or mAU*min) under the peak can be used to compare the relative abundance of each fraction. However, quantification comparison between samples can be challenging if the fractions are established at very different elution volumes or time. It is also possible to obtain the concentration of each fraction if they are collected, dialyzed (to remove the salts that were added with the buffer solution), freeze dried, and later weighted.

Furthermore, by utilizing the 'Gel Filtration HMW Calibration Kit (Cytiva, Product Code 28-4038-42)', a calibration curve can be constructed by plotting K_{av} values against the logarithm of molecular weights. This enables the determination of the molecular weight range for each fraction (further details can be found in Appendix L). However, note that accuracy may be affected since only proteins are employed as standards.

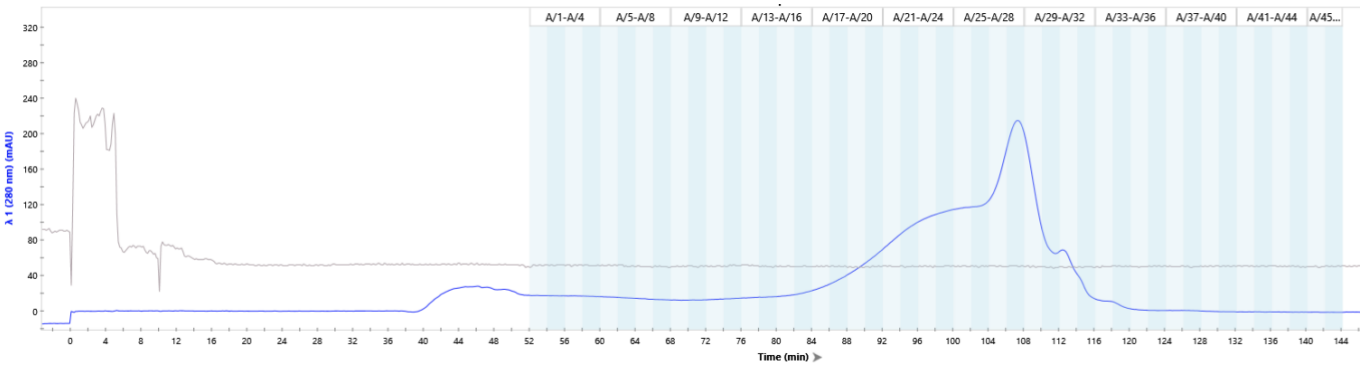

Figure 7: Example of SEC output for a Kaumera sample.

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6.6. FOURIER-TRANSFORM INFRARED SPECTROSCOPY (FTIR)

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6.6.1. INTRODUCTION

Fourier-transform infrared spectroscopy (FTIR) is an analytical technique used to identify organic and polymeric materials (RTI Laboratories, 2022). This method consists of applying infrared radiation to a sample in order to measure the sample's absorbance of infrared light at various wavelengths. The goal is to identify the material's molecular composition and structure, enabling the identification of functional groups (Mathias, 2022).

FTIR can be coupled with Attenuated Total Reflectance (ATR), which involves bringing the sample in direct contact with an ATR crystal which can effectively reflect and attenuate the infrared light (Agilent, n.d.). This interaction between the sample and the ATR crystal allows for the measurement of the sample's infrared spectrum. ATR is particularly useful for analyzing dense materials and minimizes the need for complex sample preparation (Mettler-Toledo International Inc., 2023).

FTIR-ATR has been a useful tool in analyzing Kaumera in order to identify unknown compounds and to confirm the identity of known ones. This method finds relevance in industry-specific discussions concerning Kaumera's applications, and it holds potential for integration into the extraction process for continuous quality measurements.

6.6.2. METHOD DESCRIPTION

For the FTIR-ATR analysis, it is advisable to purify the Kaumera acidic gel by dialyzing it against Milli-Q water through a 3.5 kDa membrane (see Section 5.2.). This process helps decrease the salt content and other impurities in the sample, reducing potential disruptions that might complicate the interpretation of the spectrum and make it more challenging to identify subtle characteristics or variations related to the substance of interest. Additionally, it enables a fair comparison of EPS samples from various sources that may initially have different salt concentrations. Following dialysis, the sample is freeze-dried (see Section 5.3.).

Before placing the sample on the crystal surface, the background is scanned in order to subtract the background spectrum from the sample spectrum and eliminate unwanted residual peaks. Afterwards, the sample is placed on the crystal surface and pressed down to ensure optimal contact between the sample and the surface (see Figure 8a). The FTIR instrument directs infrared radiation spanning from approximately 10,000 to 100 cm^{-1} through the sample, where some of the radiation gets absorbed, and the rest passes through (see Figure 8b). Within the sample, the absorbed radiation is transformed into rotational and/or vibrational energy by the molecules present (RTI Laboratories, 2022). This process yields a signal at the detector, manifesting as a spectrum, typically ranging from 4000 cm^{-1} to 600 cm^{-1}. Each molecule or chemical structure generates a unique spectral fingerprint, which underscores the utility of FTIR analysis in chemical identification. Depending on the desired resolution, several scans are conducted.

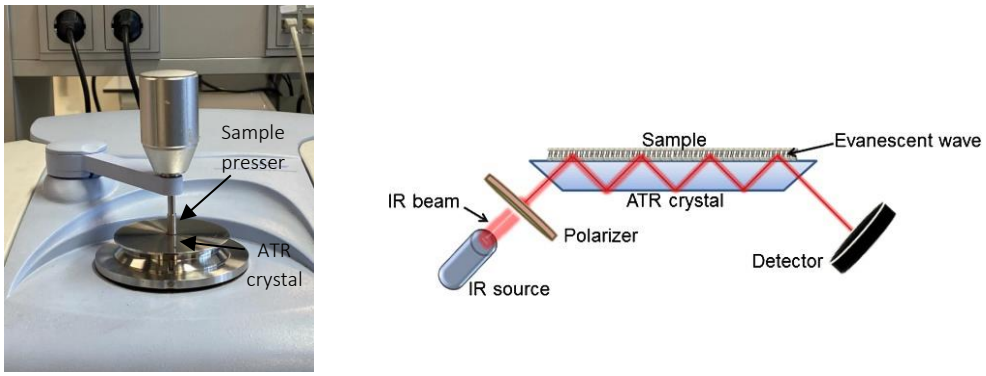

Figure 8: (a) FTIR-ATR (PerkinElmer, Shelton, USA) sample placement (b) schematic representation of an FTIR-ATR system (Ausili et al., 2015).

6.6.3. DATA ANALYSIS

FTIR-ATR data is presented as a graph where the x-axis shows the infrared spectrum, typically spanning wavenumbers from 4000 to 600 cm^{-1}, while the y-axis represents the amount of infrared light absorbed by the sample. For better visualization, the absorbances can be normalized in respect to the maximum absorbance. As illustrated in Figure 9, which shows an example of FTIR-ATR spectra for Kaumera from Utrecht and Faro, the progression along the x-axis goes from higher wavenumbers to lower wavenumbers.

The first step to interpret FTIR data involves inspecting the peaks within the spectrum. Each peak corresponds to a specific vibrational mode within a molecule, which typically arises from the stretching or bending of chemical bonds. These vibrational modes are associated with the presence of specific functional groups within the molecule. Therefore, it is possible to compare the spectrum with reference spectra or databases to determine the functional groups in the sample based on the wavenumber at which the peak is situated. As an example, see Figure 9 and Table 3. Each peak was assigned according to "Advances in Fourier Transform Infrared (FTIR) Spectroscopy of Biological Tissues" (Talari et al., 2016).

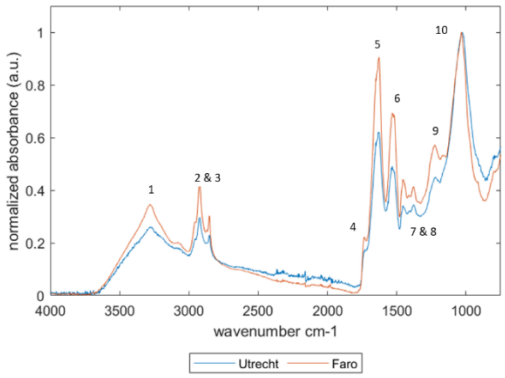

Figure 9: Absorbance spectra of Kaumera from Utrecht and Faro.

Table 3: Functional groups in Kaumera from Utrecht and Faro based on wavenumber.

Peak #	Wavenumber (cm^{-1})	Functional Groups
1	3300	Amide A (N-H stretch) in proteins and nucleic acids
2	2925	C-H stretch
3	2850	C-H stretch
4	1730	Ester
5	1630	Amide I
6	1530	Amide II
7	1455	Methyl (proteins)
8	1380	Methyl (proteins)
9	1220	Tertiary amine (C-N stretch)
10	1030	Primary alcohol (C-O stretch)

In FTIR-ATR it is also possible to obtain data from the ratio between functional groups. For example, when peak #5 (Amide I, linked to proteins) is qualitatively compared to peak #10 (primary alcohol, associated with polysaccharides) for Kaumera from Utrecht and Faro, it can be concluded that the protein-to-carbohydrates ratio is higher for Faro than for Utrecht. Moreover, FTIR data can be correlated or verified with titration data in the identification of functional groups (see Section 6.11.).

Furthermore, it is essential to consider the overall shape of the FTIR spectrum and interpret the results within the specific context of the experiment or analysis being conducted. Also note that a successful interpretation depends not only on the presence of specific bands in the spectrum but also on the absence of other significant bands (Coates, 2000).

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6.7. HEAVY METALS

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6.7.1. INTRODUCTION

Heavy metals are metallic elements characterized by their higher density compared to water (Fergusson, 1990). It is commonly assumed that the heaviness of these elements is associated with their toxicity, as they can induce toxicity even at low exposure levels (Duffus, 2002). In recent years, there has been growing concern about ecological and global public health issues related to the contamination of the environment by these metals (Tchounwou et al., 2012). Human exposure has seen a significant increase due to their widespread use in various industrial, agricultural, domestic, and technological applications. The sources of heavy metals in the environment include natural occurrences (geogenic), industrial activities, agriculture, pharmaceuticals, atmospheric deposition, and domestic waste (He & Stoffella, 2005).

Heavy metals are categorized as trace elements due to their presence in minimal concentrations (ranging from parts per billion to less than 10 parts per million) in different environmental matrices (Kabata-Pendia & Pendias, 1992). In low concentrations, heavy metals such as cobalt (Co), copper (Cu), chromium (Cr), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se), and zinc (Zn), are essential nutrients required by organisms for a range of biochemical and physiological processes (WHO/FAO/IAEA, 1996).

Kaumera holds promise for applications in agriculture, either as a biostimulant or for coating fertilizers. Consequently, measuring heavy metals is essential in order to adhere to national guidelines regulating their use, which ensure that crops and human health remain safeguarded from potential risks. Table 4 displays the average metal concentrations for Kaumera obtained from Vroomshoop, Epe, and Dinxperlo (STOWA, 2019).

Table 4: Average metal composition of Kaumera from Vroomshoop, Epe, and Dinxperlo WWTPs (STOWA, 2019).

Metal	Concentration (mg/kg DS)
Iron	7000 - 10000
Calcium	4000 - 5000
Aluminum	3000 - 5000
Zinc	400 - 1000
Copper	100 - 500
Lead	28 - 71
Cadmium	0.5 - 1
Arsenic	2 - 3

6.7.2. METHOD DESCRIPTION

Both Inductively Coupled Plasma – Optical Emission Spectrometry (ICP-OES) and Inductively Coupled Plasma – Mass Spectrometry (ICP-MS) are commonly used techniques for analyzing trace elements. ICP-OES quantifies elements by measuring the emission of excited atoms and ions at their characteristic wavelengths (Radboud University, n.d.). In contrast, ICP-MS determines the mass of atoms using mass spectrometry (MS). This distinction in the detection of metal elements results in ICP-MS having a lower detection limit that can reach parts per trillion (ppt), while ICP-OES typically achieves detection limits in parts per billion (ppb). Therefore, if the desired elements have expected

concentrations that are below or near the lower detection limit of ICP-OES, ICP-MS becomes the preferred analytical instrument.

Before subjecting Kaumera samples to ICP analysis, a preliminary step involving microwave-assisted acid digestion is necessary. This process entails exciting nearby water molecules to disintegrate the sample materials, with the aid of acids to speed homogenization (De Palma, 2020). In this way, the elements are present in solution with uniform oxidation states, making it suitable for analysis via ICP-OES/MS. Following digestion, the samples undergo filtration using a 0.2-micrometer filter to remove particulate matter remnants in the digested liquid, as this could lead to blockages of the nebulizer. Afterwards, the samples are diluted to achieve the desired volume, ensuring that the concentration of the elements falls within the ICP's operational range. Commonly, the final solution requires 1% (v/v) nitric acid to keep metals in solution of ICP-OES/MS testing. Figure 10 provides an overview of the recommended steps to be followed.

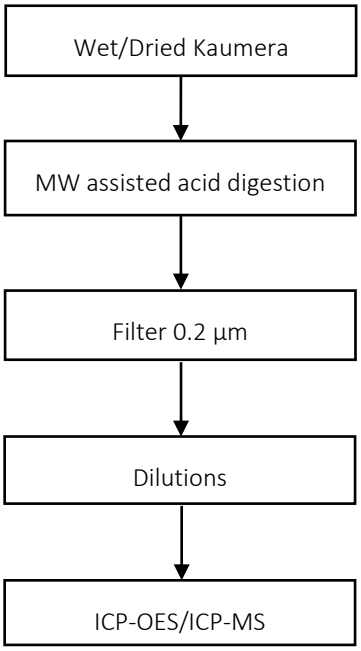

Figure 10: Scheme to measure heavy metals in Kaumera samples.

6.7.3. PROCEDURE

When measuring heavy metal concentrations in Kaumera samples in the Netherlands, the standard analysis follows the guidelines outlined in NEN 6950, encompassing digestion in accordance with NEN 6961 and measurement in accordance with NEN-EN-ISO 17294-2.

6.7.4. MICROWAVE (MW) ASSISTED ACID DIGESTION

A representative sample is dissolved in concentrated nitric acid (69% HNO_3) or in a mixture of concentrated acids using microwave heating with an appropriate laboratory microwave unit. There is no single acid or acid mixture that is universally applicable, and the choice of an acid or acid mixture to be used depends on the analytes of interest. Nevertheless, it is imperative that the digestion is conducted thoroughly and that no visible solids remain after

digestion. The sample, along with the acid(s), is introduced into a vessel made of fluorocarbon polymer (PFA or TFM) or quartz. The vessel is securely sealed and then subjected to microwave heating for a specified duration.

6.7.5. INDUCTIVELY COUPLED PLASMA – OPTICAL EMISSION SPECTROMETRY (ICP-OES)

ICP-OES is a technique employed for the elemental analysis of liquid samples by utilizing a plasma and a spectrometer. The sample is introduced into the system through a peristaltic pump, accompanied by a carrier solution (HNO_3) and an internal standard (e.g., Yttrium) (Radboud University, n.d.). Within the system, the sample is nebulized within a spray chamber, forming an aerosol that is subsequently injected into an argon plasma. During this process, the sample undergoes atomization and ionization.

As a result of this treatment, the sample's electrons absorb thermal energy, causing them to transition to a higher "excited" state (Radboud University, n.d.). When these electrons return to their ground state, they release energy in the form of light or photons. Each element exhibits its own unique emission spectrum, which is detected and measured by the spectrometer. The intensity of light at specific wavelengths is recorded and following calibration, converted into concentration values.

6.7.6. INDUCTIVELY COUPLED PLASMA – MASS SPECTROMETRY (ICP-MS)

In ICP-MS the sample solution is introduced into the instrument using a peristaltic pump (Radboud University, n.d.). It undergoes nebulization in a spray chamber, forming an aerosol. This aerosol is then injected into an argon plasma with a temperature ranging from 6000-8000 K. The high temperatures cause the atoms in the sample to undergo ionization and, to some extent, atomization. This means that electrons are stripped from the atoms, creating positively charged ions. The resulting ions are then accelerated into the mass spectrometer, where a magnetic or electric field is used to separate the ions based on their mass-to-charge ratio (m/z). Afterwards, the ions pass through the detector, and the signal produced is proportional to the abundance of each ion. The detector signal is then analyzed, and the concentrations of the elements or isotopes in the original sample are determined based on calibration with standards of known concentration.

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6.8. PATHOGENS

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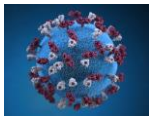
6.8.1. INTRODUCTION

Pathogens are microorganisms that cause disease or illness to their host organisms. They can be classified into various categories, including bacteria, viruses, and protozoa (see Table 5). The major sources for pathogenic organisms are animal feeding operations, biosolids, on-site treatment systems, and municipal wastewater treatment plant effluents (Gerba & Smith, 2005).

Kaumera is sourced from municipal or industrial aerobic granular sludge. In the case of municipal sludge, it can potentially harbor pathogens commonly found in municipal waste, such as E. coli, Enterococci, and Clostridia (STOWA, 2019). Nevertheless, it has been demonstrated that these microorganisms are effectively eradicated during the Kaumera production process, primarily through the introduction of high-temperature water and/or chemical treatments. However, if Kaumera is intended for use in applications such as agriculture or those involving human contact, it is essential to verify pathogen concentrations to ensure compliance with regional guidelines.

When the goal is to measure the presence of waterborne pathogens, a significant challenge arises due to their sheer quantity and diversity. Consequently, it is common to focus on quantifying indicator organisms as a more manageable and practical approach (EPA, 2023). These organisms indicate the presence of fecal material in the sample and, therefore, of the possible presence of disease-causing bacteria, viruses, and protozoa. Indicator organisms are typically not pathogenic, exhibit limited growth in water, and are reliably detectable even at low concentrations (Motlagh & Yang, 2019). Ideally, the indicator organisms should exhibit survival rates closely mirroring those of the actual pathogens they represent, and they should also be present in greater quantities than the targeted pathogens. Some examples of indicator organisms include somatic bacteriophages, E.coli, spores of sulfite reducing clostridia (SSRC), and Enterococci.

Table 5: Categories of waterborne pathogens (EPA, 1999).

Category	Size (µm)	Description	Examples	Image
Virus	0.01-0.1	Composed of DNA or RNA surrounded by a protective protein coat	Adenovirus, Hepatitis A, Rotavirus	
Bacteria	0.1-10	Single-cell prokaryotes	Vibrio cholera, Enterococci, Clostridium perfringens spores, E. coli	
Protozoa	1-20	Single-cell eukaryotes	Cryptosporidium, Giardia lamblia, Entamoeba histolytica	

6.8.2. METHOD DESCRIPTION

Counting microorganisms involves a systematic process, commencing with sample collection, preparation, and often serial dilution to reduce the concentration of microorganisms. Samples are then plated on appropriate growth media,

such as agar, and incubated under controlled conditions (temperature and time), allowing microorganisms to form visible colonies. These colonies are subsequently counted manually or with the aid of automated tools, and the results are expressed as colony-forming units (CFUs) per unit volume or weight, depending on the sample type. Quality control measures are essential to ensure the reliability and precision of the microbial counts, with variations in the procedure based on microorganism type, sample nature, and research objectives.

6.8.3. PROCEDURE

Kaumera samples are analysed for Clostridium perfringens and its spores at a temperature of 37°C, following the guidelines outlined in ISO 7937. Enumeration of Enterococci at the same temperature is carried out in accordance with NEN-EN 15788. The enumeration of Escherichia coli is performed at 44°C, adhering to the specifications of ISO 16649-2.

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6.9. YIELD STRESS (RHEOMETER) AND INTRINSIC VISCOSITY

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6.9.1. INTRODUCTION

In laboratory settings, a rheometer can be used to gather data on the yield stress and intrinsic viscosity of Kaumera, in contrast to the on-site usage of the Brookfield DV2T Viscometer, as outlined in Section 7.3. Since the latter yields purely qualitative results, it cannot be directly compared to the quantitative data obtained with a rheometer.

The yield stress and the intrinsic viscosity offer insights into Kaumera's gel-forming properties, even without detailed knowledge of its chemical composition. These measurements are valuable for understanding the ease of polymer extraction and have practical relevance in various industrial applications, including seed coatings, composites, and more. Additionally, intrinsic viscosity values provide a measure for the size of the individual polymer coils and thus, considerable insight about changes to the polymer coils' conformations due to variations in pH, conductivity, counterions, and temperature. It can further be used along with the Mark-Houwink-Sakurada equation to obtain the molar mass of the polymer coils.

6.9.2. DEFINITIONS

The "yield stress" is defined as the minimum stress that must be applied to a sample before it starts to flow (Lee et al., 2015). "Intrinsic viscosity" measures the size of the polymer coils in solution (Lu & Mays, 2021), and it is dependent on the polymer-solvent v/s polymer-polymer interactions. It is possible to calculate intrinsic viscosity with the "relative viscosity", a fundamental measure of solution viscosity (see Table 6). For highly concentrated systems, the relative viscosity may also be calculated using the "consistency index" (κ in place of η), a parameter from the Herschel-Bulkley model (1926) for non-Newtonian fluids. In fact, the same Herschel-Bulkley model may also be used to calculate the yield stress and consistency index simultaneously.

Table 6: Equations for the different solution viscosities.

Parameter	Unit	Equation
Relative Viscosity	(-)	$\eta_r = \frac{\eta}{\eta_0}$
Specific Viscosity	(-)	$\eta_{sp} = \eta_r - 1 = \frac{(\eta - \eta_0)}{\eta_0}$
Intrinsic Viscosity	dL/g	$\eta_{int} = \lim_{C \to 0} \frac{\eta_{sp}}{C}$

6.9.3. METHOD DESCRIPTION

It has been observed that removing inorganic substances such as salts improve the rheological properties of Kaumera. Hence, to minimize impurities and inorganic substances in the samples, an initial washing with HCl (Section 5.1.) or a dialysis step using a 3.5 kDa MWCO membrane against Milli-Q water (Section 5.2.) can be performed.

By diluting, separate samples are prepared, each with a different concentration (TS%). During rheometer testing, a stress control measurement setting is employed, with stress levels transitioning from high to low values in a linear trend. A conditioning ramp is conducted in order to homogenize the sample. Afterwards, three stress ramps, each featuring specific waiting periods (10, 100, and 1000 seconds), are conducted to assure there are no major structural

changes over time. From the rheometer, plots of shear rate (1/s) vs. shear stress (Pa) are obtained. To find the values for yield stress and consistency index (parameter necessary to calculate intrinsic viscosity), the *scipy.optimize.curve_fit* function in Python can be used to fit data to the Herschel-Bulkley model. The y-intercept of the curve equals the yield stress. The consistency index calculated for each concentration (TS%) is related to the specific viscosity, and when this is plotted against concentration, the slope equals the intrinsic viscosity. The intrinsic viscosity is defined as the initial slope, taking the limit $C \rightarrow 0$.

6.9.4. PROCEDURE

The detailed procedure can be found in Appendix M.

6.9.5. DATA ANALYSIS

The primary focus among the rheometer's various outputs is the 'shear rate vs. shear stress' plot. Typically, this data is reversed and displayed with 'shear stress (Pa)' on the y-axis and 'shear rate (1/s)' on the x-axis, as illustrated in Figure 11.

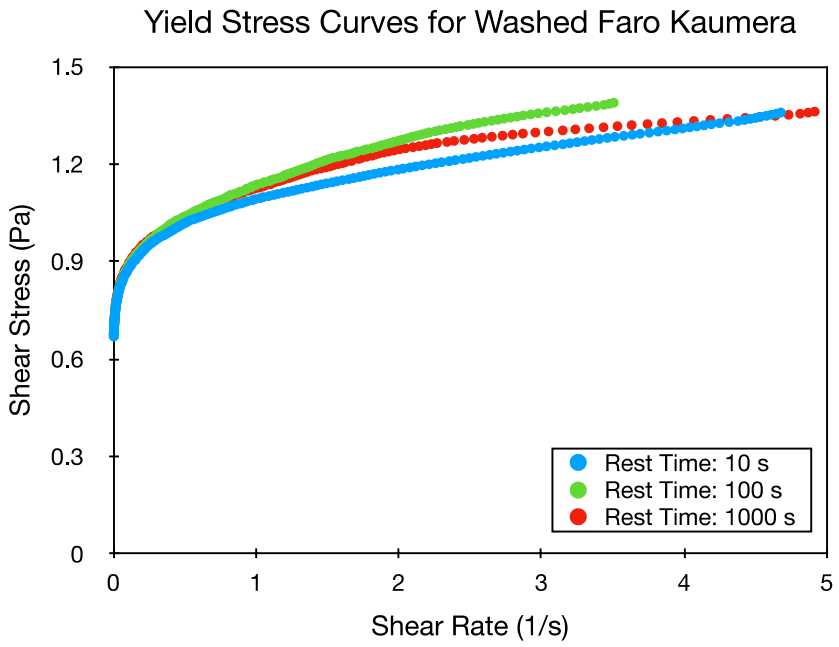

Figure 11: Yield Stress Curves for Washed Kaumera from Faro at different rest times.

To find the values for yield stress and consistency index, the data can be fitted to the Herschel-Bulkley model with the function *scipy.optimize.curve_fit* in Python. The constitutive equation of the Herschel-Bulkley model for a fluid can be written as follows:

$$\tau = \tau_0 + \kappa \dot{\gamma}^n$$

Where τ is the shear stress (Pa), τ_0 the yield stress (Pa), κ the consistency index (Pa), $\dot{\gamma}$ the shear rate (-),and n is the flow index (-).

τ and $\dot{\gamma}$ are known, while three unknowns remain (τ_0, κ, and n). Thus, the curve is best-fitted to obtain the missing parameters. It will be necessary to provide initial guess values for the parameters τ_0, κ, and n. As a rule of thumb, $0 < n < 1$, as the fluid is shear-thinning.

For each sample with a given %TS, the average yield stress is calculated as the mean yield stress, along with the standard deviation, derived from the three datasets obtained with the different waiting periods (10, 100, and 1000 seconds). In the case of the concentric cylinder geometry however, the average consistency index is obtained from the plateau value of the viscosity.

Once the average consistency index is obtained for each sample (%TS), the intrinsic viscosity can be calculated at very low concentrations where the polymer coils don't overlap or don't substantially interact with each other, and a linear trend is observed. With the equation in Table 6 the relative consistency index can be calculated (at low concentrations, the solution is Newtonian, meaning n = 1 in the Herschel-Bulkley model, and thus $\kappa = \eta$). The relative consistency index is then plotted against concentration (TS%) to obtain the slope, which is equal to the intrinsic viscosity. An example is presented in Figure 12.

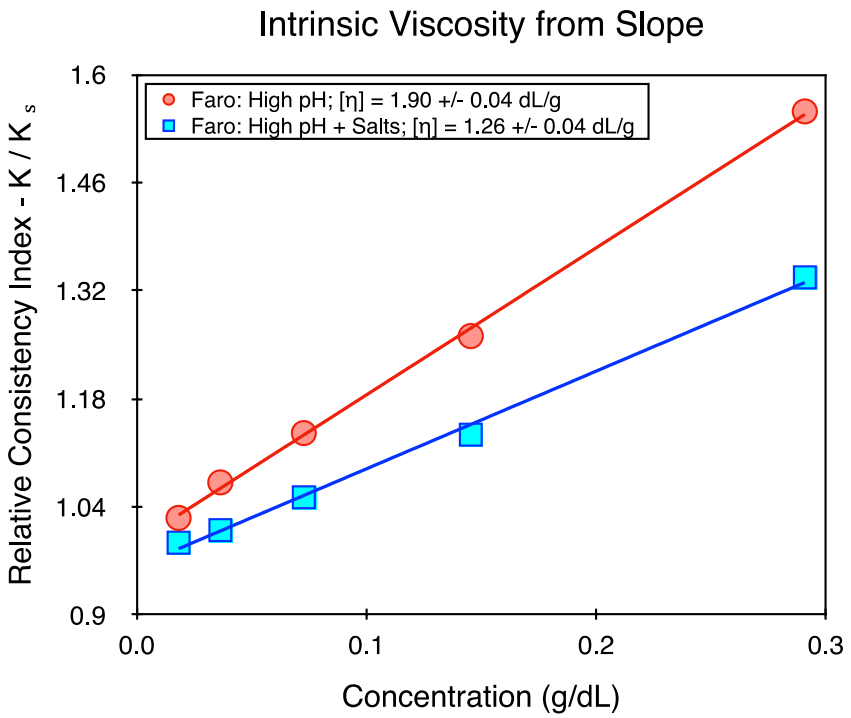

Figure 12: Intrinsic Viscosity measured for washed Kaumera samples from Faro.

6.9.6. ADDITIONAL CONSIDERATIONS

When employing a rheometer to analyze Kaumera, it is important to note that the data obtained may exhibit more noise than that of other biopolymers. To mitigate this, it is possible to conduct multiple measurements. Run the system repeatedly to achieve the desired curves, ensuring that the applied shear stresses and shear rates fall within acceptable ranges. If the goal is to assess multiple Kaumera samples, it is crucial to determine whether they can genuinely be compared. If significant variations exist, such as in the extraction process, the materials will diverge significantly from one another, making them non-comparable.

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6.10. THERMOGRAVIMETRIC ANALYSIS (TGA)

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6.10.1. DEFINITIONS

As defined by the American Public Health Association (APHA, 1999):

- Total solids (TS): material residue left in the vessel after evaporation and drying in an oven at 105°C.
- Volatile solids (VS):The weight that is lost on ignition in a muffle furnace at 550°C.
- Pyrolysis: chemical decomposition of organic materials by heating in the absence of oxygen or any other reagents.

6.10.2. INTRODUCTION

Thermogravimetric analysis (TGA) is a characterization test that involves measuring a sample's weight as the temperature increases at a constant heating rate. TGA is a valuable tool for assessing the thermal stability of materials (Ebnesajjad, 2006). Observing changes in the sample's weight can provide insights into ongoing processes, including dehydration, oxidation, or decomposition (Coats & Redfern, 1963). In addition, TGA can determine total solids (TS) and volatile solids (VS), offering a faster alternative to the process described in Section 6.1. When analyzing Kaumera, TGA can also help examine its thermal resistance, as it can reach temperatures of up to 1200°C.

Moreover, when TGA is coupled with other complementary analytical techniques such as mass spectrometry (MS), gas chromatography (GC), or Fourier-transform infrared spectroscopy (FTIR), it can provide insight into the chemical composition and the presence of specific compounds in a material (Abraham et al., 2018).

A basic TGA apparatus consists of four components: a balance, a sample pan, a furnace, and a purge gas system. The balance is highly sensitive and capable of accurately measuring the sample's weight at specific time intervals. The balance is positioned above the furnace and is thermally isolated from the heating element. The pan is where the sample is loaded and unloaded, while the programmable furnace is responsible for heating the sample to the desired temperature. A purge gas system is employed to allow the flow of gas over the sample, flushing out any vaporized sample or decomposition products. This can involve the use of gases like nitrogen, helium, air, or even the creation of a vacuum environment.

6.10.3. METHOD DESCRIPTION

In the TGA analysis process, a small sample of approximately 25 µL of acidic gel Kaumera is placed onto the sample pan. The choice of heating rate and time intervals depends on the specific purpose of the analysis. For instance, if the main goal is to exclusively measure the total solids in the sample, opting for a high heating rate can rapidly reach 105°C. Conversely, using a lower heating rate during specified time intervals enables a more detailed examination, allowing for the observation of precise weight changes at specific temperatures which may provide clues on thermally activated processes occurring in the sample.

Moreover, it is common to use nitrogen as the purging gas before reaching 550°C. This is because oxygen can cause the sample to oxidize while nitrogen is an inert gas. This approach is used to ensure that the sample only reacts to temperature during decomposition (pyrolysis). After reaching 550°C, the nitrogen atmosphere can be switched to oxygen or air. However, if the goal is to compare the TGA data with results obtained using the 105°C and 550°C furnace methods (Section 6.1.), it is recommended that the entire procedure is conducted using air.

6.10.4. DATA ANALYSIS

Usually, the TGA graph (or weight loss curve) displays the weight (mg) or weight change (%) on the y-axis and temperature or time on the x-axis. Alternatively, it may also represent time on the x-axis and employ a dual y-axis setup, with weight change (%) and temperature, as illustrated in Figure 13. The TGA thermal curve is plotted from left to right, and a descending section in the curve indicates a decrease in weight.

An important analysis involves calculating the first derivative of the TGA curve, which is referred to as the Differential Thermal Gravimetry (DTG) curve. The DTG curve provides information about the rate of change of the sample's weight with respect to temperature. The peaks on a DTG curve indicate the points of greatest rate of change on the weight loss curve, also known as inflection points. These peaks represent specific thermal events such as decomposition or phase transitions and can be particularly useful for pinpointing the temperature at which these events occur.

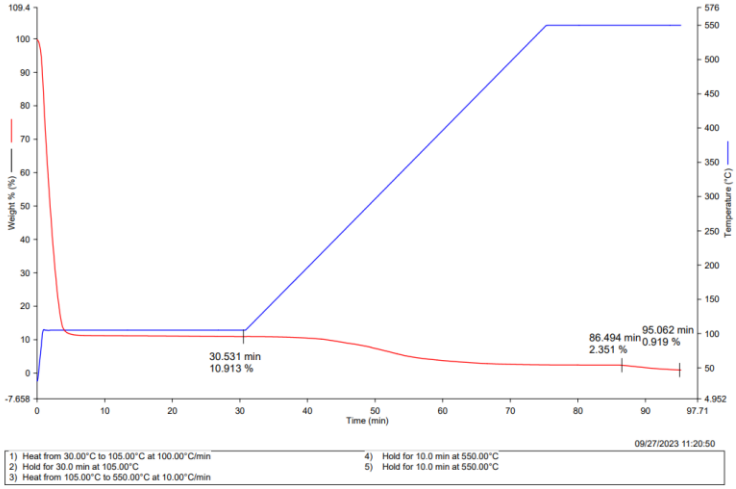

Figure 13: TGA example procedure and data on 12% TS washed Kaumera Faro.

6.10.5. ADDITIONAL CONSIDERATIONS

- To ensure reproducibility, use approximately the same sample weight during each experiment.
- Clean the TGA furnace tube/sample pan and conduct the calibration routines as suggested in the user manual.

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6.11. ACID-BASE AND CONDUCTOMETRIC TITRATION

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6.11.1. DEFINITIONS

- Strong acid/base: any acid/base that ionizes completely in solution.
- Weak acid/base: any acid/base that ionizes partially in solution.
- Equivalence point: point at which the moles of titrant added equal the original moles of analyte in the solution.
- Half-equivalent point: for a weak acid titration, it is the point at which half of the moles of the weak acid have reacted with the strong base. At this point, pH ≈ pKa.
- Polyprotic acid or base: substance capable of donating or accepting multiple protons per molecule.
- Zwitterion: chemical species that contain both positive and negative charges within the same molecule. As a result, it can act as both an acid and a base in a chemical reaction.
- Charge density: amount of electric charge per unit (mass or as a mole fraction).

6.11.2. INTRODUCTION

Acid-base and conductometric titration is a characterization method that provides information about the polymer's acid-base properties, ionization behavior, and charge distribution. This includes the possibility to determine pKa values of the acidic and basic groups, identify and quantify functional groups, and calculate the charge density.

The information gathered from this method can be cross-referenced with and related to results from other characterization techniques. For example, the charge density values obtained using this method can be compared with those obtained through electrophoretic and light scattering techniques (Section 6.12.). Additionally, by jointly assessing the titration and FTIR data (Section 6.6.), it becomes possible to determine the role of different functional groups.

6.11.2.1. ACID-BASE TITRATIONS

Acid-base titration involves the gradual addition or removal of protons. In its simplest form, this process employs a burette to deliver precisely measured volumes of a known concentration of acid or base solution (titrant) into a flask containing an unknown concentration of a base or acid (analyte) (Jennings et al., 2010). For example, consider the neutralization of a strong or weak acid with a strong base (e.g., NaOH). As the base is introduced, the pH of the solution gradually rises. At a specific moment, the equivalence point is reached, which is when the moles of the added base equal the initial moles of acid in the solution (free H_3O^+ is converted to $2H_2O$ with OH^-). This usually coincides with the midpoint of the rapid pH increase. In the case of acidified EPS, a second equivalence point occurs when the analyte is completely deprotonated by OH^- (leading to an increase in conductivity due to the excess OH^-). The segment of interest lies between these two equivalence points, representing the phase where the added OH^- reacts with the analyte.

The shape of the pH curve in acid-base titrations varies depending on the strength of the acids involved. Strong acid titrations produce an S-shaped curve, while weak acid titrations result in a more gradual pH change near the equivalence point (Jennings et al., 2010). Since weak acids only partially dissociate, their pH behavior is influenced by both their pKa and concentration. The pKa of a weak acid can be determined at the half-equivalence point, offering valuable insights into its properties. Furthermore, at the equivalence point, weak acid titrations result in a more basic solution (pH>7), whereas strong acid titrations reach the equivalence point at pH 7.

In the case of a polyprotic acid or base, the neutralization reaction occurs in stages (Young, 2018). It starts with the titration of the most acidic group, followed by the next acidic group, and so on. This sequential process results in a series of inflection points within the titration curve, where the pH equals the pKa of each specific group.

Some other species can also behave as zwitterion or dipolar ion. Amino acids are zwitterions as they have at least two ionizing groups: -COOH and -NH_3^+ (Lehninger et al., 2005). In this case, the former dissociates more easily than the latter. For example, consider the amino acid glycine in the context of a titration curve (see Figure 14). Initially, at the start of the curve, glycine is protonated, carrying a positive charge attributed to the NH_3^+ group. The first dissociation point, marked as pK_1 and occurring at around pH 2.34, results due to deprotonation of the COOH groups. At the equivalence point with a pH of 5.97, neutrality is reached, as COO- groups now balance out the positive NH_3^+-charges. This point is also known as the isoelectric or electrically neutral point (pI). Further along the curve, the second dissociation point is observed, referred to as pK_2 with a value of 9.60. At this stage, the NH_3^+ groups undergo deprotonation, leading to a net negative charge for the compound. Every amino acid possesses a unique combination of pK and pI values specific to its chemical properties.

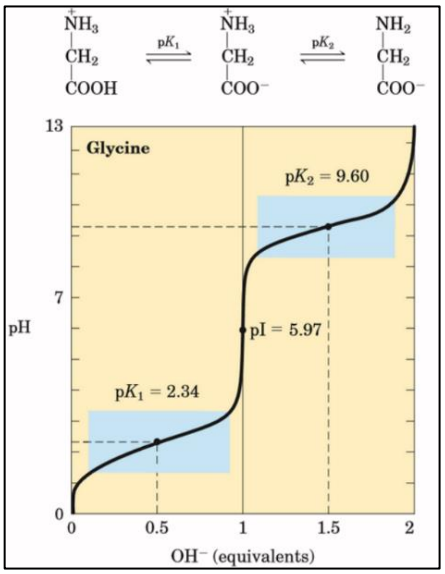

Figure 14: Glycine titration at 25°C (Lehninger et al., 2005).

6.11.2.2. CONDUCTOMETRIC TITRATIONS

Conductometric titrations are used to examine changes in conductivity resulting from neutralization, ion exchange, precipitation, and complex formation reactions (Zhao et al., 2017). The underlying principle centers on the replacement of one type of ion by another (Young et al., 2018), and these ions typically exhibit different mobilities (Table 7). Consequently, the solution's conductivity undergoes variations as the titrant volume changes. Just before and after the conductivity turning point, which signifies the equivalence point, there is a noticeable difference in the rate of conductivity change with respect to the titrant volume. The detection of a turning point in the conductivity slope allows for the determination of the volume of titrant required to reach equivalence with the analyte solution. In the context of a neutralization reaction, this information enables the calculation of equivalent molarity relative to the mass of the analyte. This allows the calculation of charge density, which quantifies the distribution of charged groups (either positive or negative) along the molecular backbone of a biomacromolecule.

Table 7: Mobilities of different ions in water at 298 K (Atkins et al., 2014).

Ionic mobilities in water at 298 K, ($u/(10^{-8}m^2s^{-1}V^{-1})$)			
Cation	Mobility	Anion	Mobility
H_3O^+	36.23	OH^-	20.64
K^+	7.62	Br^-	8.09
NH_4^+	7.62	I^-	7.96
Ca^{2+}	6.17	Cl^-	7.92
Na^+	5.19	NO_3^-	7.41
Li^+	4.01	F^-	5.74

A straightforward example is the titration of a strong acid with a strong base such as NaOH. Initially, the acid solution exhibits high conductivity because of the abundance of mobile H_3O^+ ions. When the base is introduced, each H_3O^+ ion is replaced by Na^+ ions, which have significantly lower ionic mobility (7 times less than H_3O^+), resulting in a decrease in conductivity as water is formed. This decline continues until the equivalence point is reached. Further addition of the base introduces excess OH^- ions, causing the conductivity to increase once more. The distinct behavior of weak electrolytes is primarily attributed to their lower degree of ionization, as their chemical equilibria shift differently. For example, NH_4^+ salts, being less prone to ionization, exhibit a conductivity plateau.

In a general context, when dealing with low-viscosity solutions containing fully ionized electrolytes, conductivity is closely linked to the size of ions, particularly their hydrodynamic radius, which reflects their distinct rates of movement in an electric field (Atkins et al., 2014). Increasing the viscosity of the solution introduces greater resistance to movement, leading to a reduction in overall conductivity. Similarly, changes in solution temperature have a comparable but more intricate impact, particularly concerning the performance of the conductivity cell electrodes.

6.11.3. CONSIDERATIONS

- It is a recommended to perform a separate titration of the titrant solution to verify its molarity rather than solely relying on the information provided on the bottle by the chemical company. This can be done with a standard solution.
- Specific acid-base reactions can occur in the presence of carbon dioxide (CO_2), which dissolves in water, forming carbonic acid (H_2CO_3). This compound functions as a buffer and can impact data accuracy. To prevent CO_2 dissolution, bubbling nitrogen (N_2) gas into the analyte is recommended. This acts as a N_2 blanket on top of the sample.
- Excess salts present in polymer solutions will influence the titration curve. Therefore, it is necessary to either eliminate or assess these salts. Salts can be removed via the treatment step of washing (Section 5.1.) or dialysis (Section 5.2.).
- Acid-base titrations may experience decreased accuracy when the total polymer concentration is very low, approaching that of deionized or distilled water. This limitation is influenced by the specific electrode probe utilized. Additionally, if the pKa values of the compounds being titrated and their initial concentrations are known, it is possible to predict acid-base titrations accurately using pH calculation formulas found in most physical chemistry textbooks.
- Conductometric titrations may exhibit reduced accuracy when the total polymer concentration is high, especially as it approaches around 30 mS/cm. This limitation is associated with both the electrode in use and highly concentrated electrolyte solutions, which can reach a molar conductivity plateau or even lead to salt phase separation (precipitation). It is important to recognize that only strong electrolytes fully ionize and exhibit a linear relationship (square root) with concentration at low concentrations. The contribution of salts to molar conductivity varies based on their degree of ionization and the number of ions per formula unit.
- Taking the aforementioned factors into account, an optimal experimental setup should include titration curves for: (i) a blank titration without any polymer, (ii) high electrolyte or polymer concentrations, and (iii) lower electrolyte or polymer concentrations.

6.11.4. PROCEDURE

The detailed sample preparation method, experimental setup, and procedure can be found in Appendix N.

6.11.5. DATA ANALYSIS

Figure 15 illustrates the acid-base and conductometric titration curve for a Kaumera with a concentration of 0.005 M. The x-axis represents the added titrant volume (ml), while the y-axis features a dual arrangement, with pH and conductivity (measured in mS/cm).

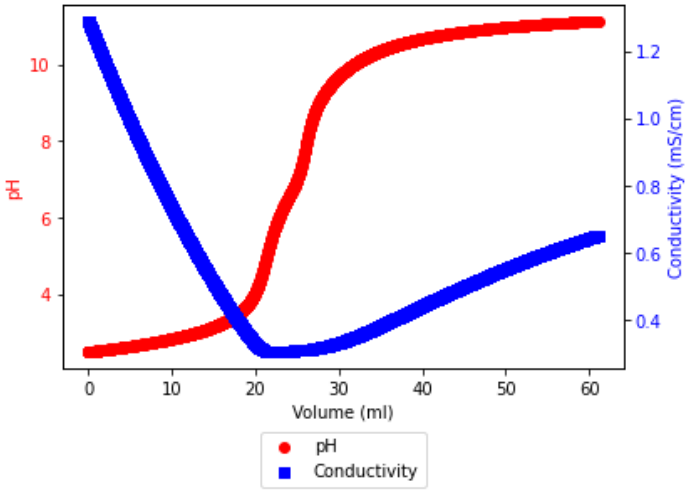

Figure 15: Acid-base and conductometric titration of a 0.005 M Kaumera.

Using the titration data, it becomes possible to identify functional groups within a molecule, as each functional group generally possesses its distinct pKa (see Figure 16). The variability in pKa values among different functional groups is attributed to their chemical structure and the local environment within the molecule. For instance, carboxylic acid groups (COOH) typically exhibit pKa values in the range of 2-5, while amino groups (NH_2) tend to have pKa values around 9-11 (Lehninger et al., 2005). Additionally, this data allows for the establishment of ratios between acidic and basic groups, which can be correlated with FTIR data.

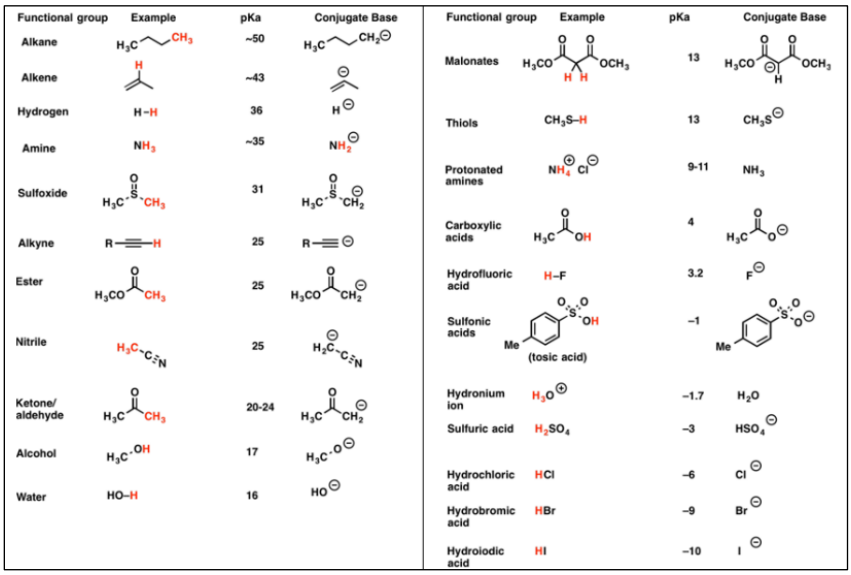

Figure 16: pKa values of different functional groups.

Furthermore, the charge density of the polymer can be assessed with the plot of measured ionic conductivity against volume of titrant (Farris et al., 2011). The intersection points of the linear segments in the ionic conductivity plot before and after reaching the equivalent point can be used to visually determine the volume (in milliliters) of titrant needed to fully neutralize the acid groups along the polymer backbone. By multiplying this volume by its concentration (molarity) and referencing the initial mass of the polymer, it becomes possible to calculate the charge density of the polymer (in millimoles per gram, $mmol.g^{-1}$).

Below is an example calculation of the charge density of pectin, a negatively charged polysaccharide (Farris et al., 2011). In this example (see Figure 17), 0.1 M NaOH was used to titrate 0.2 grams of pectin (the quantity of pectin dispersed in water to create the initial diluted pectin dispersion). To determine point A and B, a system of equations defining lines 1, 2, and 3, are applied.

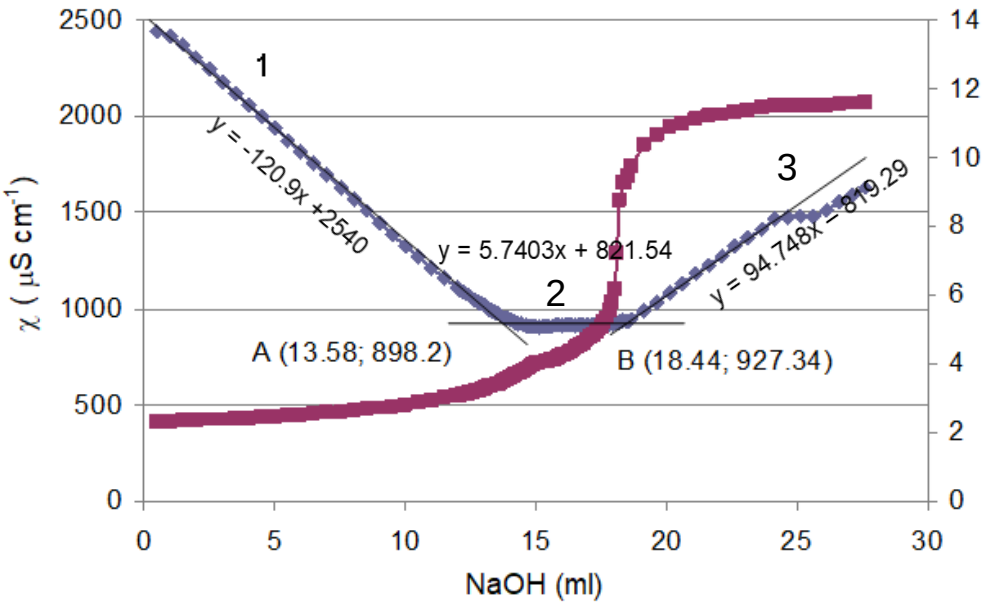

Figure 17: Example titration of 0.2 g pectin with 0.1 M NaOH (Farris et al., 2011).

The two systems are:

$$A)\begin{cases} y = -120.9x + 2540 \\ y = 5.74x + 821.5 \end{cases} \qquad B)\begin{cases} y = 94.75x - 819.3 \\ y = 5.74x + 821.5 \end{cases}$$

Which result in the coordinate values:

A (13.58, 898.2); B (18.44, 927.34)

The total amount of volume of titrant required to fully neutralize the acid groups along the pectin backbone is given by the difference:

$$18.44 - 13.58 = 4.86 \text{ ml}$$

Considering the concentration of titrant 0.1 M = 0.1 mol/L = 100 mmol/L, a straightforward proportion can be employed to determine the millimoles used to titrate the acid groups in 0.2 grams of pectin:

$$100\text{mmol} : 1000\text{ml} = x : 4.86\text{ml} \rightarrow x = 0.486 \text{ mmol}$$

Finally, the mmol per gram of pectin is determined (the unit mass):

$$0.486\text{mmol} : 0.2\text{g} = x : 1\text{g} \rightarrow x = 2.43 \text{ mmol}$$

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6.12. ZETA POTENTIAL USING DYNAMIC LIGHT SCATTERING

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6.12.1. INTRODUCTION

Zeta potential is the electrokinetic potential at the surface of shear of any interface (Hunter, 2013). It is a property of any suspended particle, macromolecule, or material surface submitted to an electric field. Zeta potentials (and corresponding electrokinetic charges) can be linked to actual surface charges and other interfacial properties of the investigated system and thus be used for optimizing the formulations of (stable) suspensions, emulsions, and protein solutions, as well as for predicting particle-particle interactions and flocculation (Elimelech et al., 2013). This measurement, for instance, becomes indispensable in the optimization of films and coatings.

Kaumera is predominantly recognized as being negatively charged, although it also possesses positive charges. Nevertheless, the interaction between Kaumera and other (charged) surfaces requires more research and can be investigated by a combination of zeta potential and complementary techniques (e.g., measuring size, settling velocities) (Shakeel et al., 2020). Measuring zeta potential can easily be done using electrophoresis techniques from which the surface charge properties of Kaumera can be deduced. Understanding how the different extraction methods can impact the charge and flocculation abilities of Kaumera is of particular interest, especially concerning potential applications.

6.12.1.1. ELECTRICAL DOUBLE LAYER

When exposed to an aqueous medium, the majority of particle surfaces become electrically charged. Typically, many naturally occurring particles possess a negative charge (e.g., clay particles). Consequently, these particles tend to repel each other, resulting in stable suspensions. The properties (pH, salinity) of the suspending medium changes the surface charge of particles and hence their flocculation ability. The size and density of the obtained flocs is the main driver for their settling velocity.

The emergence of an electric charge on the particle's surface influences the arrangement of ions in the nearby interfacial area. This leads to a higher concentration of counter ions (those carrying an opposite charge to the particle) in close proximity to the surface (Hunter, 2013; Ohshima, 2006). Consequently, an electrical double layer (Debye layer) forms around each particle (see Figure 18). The inner region, known as the Stern layer, has counter-ions strongly bounded to the particle's surface, while in the outer (diffuse) region, counter-ions are less firmly associated. In this outer region, it is also possible to observe ions with the same or opposite charge as the particle's. Furthermore, inside the diffuse layer, there exists an abstract boundary called the "hydrodynamic shear plane" or "slipping plane" behind which ions and water move with the same velocity as the colloidal particle. When a particle undergoes movement, such as due to electrophoresis, the velocity of the colloidal particle is determined by the value of the electrokinetic potential at this shear plane, which is called the zeta potential.

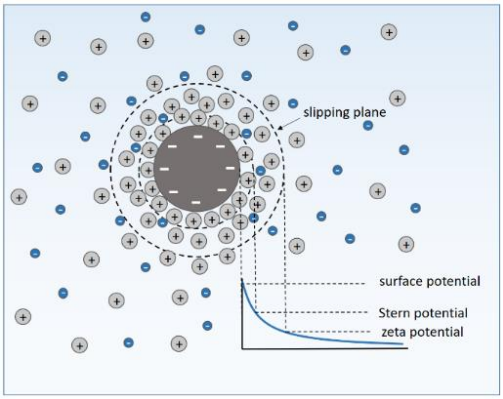

Figure 18: Graphic representation of electric double layer (Chassagne, 2021).

6.12.1.2. DVLO THEORY

In the 1940s, scientists Derjaguin, Verwey, Landau, and Overbeek developed the DVLO theory which enables to predict the stability of colloidal systems (Chassagne, 2021). According to this theory, the stability of a colloidal system depends on the combined balance of two forces: van der Waals attractive forces and electrical double layer repulsive forces. These forces come into play as particles move closer to each other due to their Brownian motion or by hydrodynamic forces (see Figure 19). Van der Waals forces are dominant at very short separations, which implies that, provided particles can approach one another at sufficient small distances, (irreversible) aggregation will occur. In order to approach one another, the particles need to overcome an energy barrier the strength of which is a function of zeta potential, which itself is a function of the particle surface charge properties (and pH dependent) and ionic strength (salinity).

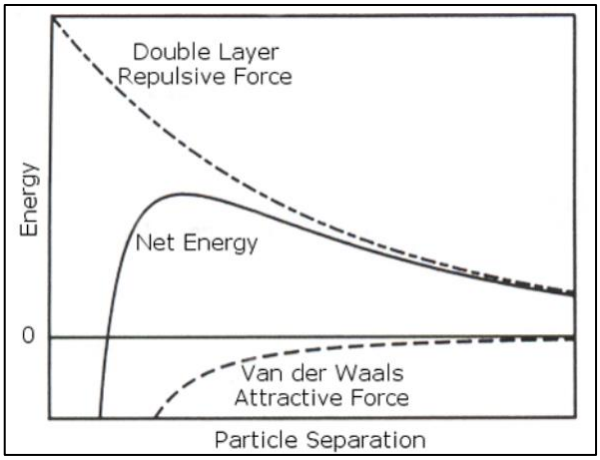

Figure 19: Schematic diagram depicting the variation in free energy as particle distance increases, as outlined by the DLVO theory.

Hence, when particles possess a substantial repulsive force, the particle-particle interaction will effectively oppose flocculation, resulting in a stable colloidal system. On the contrary, if there is a weak electrostatic repulsion, flocculation will eventually occur.

Zeta potential has been recognized as a very good index to assess the stability of a colloidal system (Verwey, 1947). When the suspended particles exhibit a significantly negative or positive zeta potential, they will repel each other, and there will be no inclination for these particles to aggregate. On the contrary, when the particles possess low zeta

potential values, there will be no counteracting force to hinder them from coming together and forming flocs. The typical threshold for distinguishing between stable and unstable suspensions is conventionally set at either +25 mV or -25 mV (corresponding to an electric potential of kT/e, whereby k is the Boltzmann constant, T the temperature (in K) at room temperature and e the absolute value of the electron charge) (Williams, 2016). Particles with zeta potentials exceeding +25 mV or falling below -25 mV are typically regarded as stable.

6.12.1.3. MEASURING ZETA POTENTIAL: MICROELECTROPHORESIS LIGHT SCATTERING

Electrophoretic laser Doppler techniques are standard techniques used to estimate zeta potentials (Hunter, 2013). In modern commercial set-ups, such as the Malvern ZetaSizer Nano, the sample is introduced into a folded capillary cell with electrodes at both ends. When the electric field is applied, charged particles within the sample move toward the electrode bearing the opposite charge, and their velocity is estimated through laser Doppler velocimetry. This technique therefore enables to estimate an average zeta potential (over the whole sample). Other equipment (as the ZetaCompact of CAD Inst.) can be used to track the mobility of a collection of particles and then the zeta potential of each tracked particle is given. In this case, video microscopy and laser sheet illumination is used to follow the particles. The particle's velocity is expressed in unit electric field strength, referred to as its electrophoretic mobility (U_E):

$$U_E = \frac{v}{E}$$

Where U_E = electrophoretic mobility, v = velocity of the particle, and E = electric field (voltage gradient).

The zeta potential is associated to electrophoretic mobility through the Henry equation:

$$U_E = \frac{2\varepsilon\zeta f(\kappa a)}{3\eta}$$

Where ζ = zeta potential, ε = dielectric constant, η= viscosity, and $f(\kappa a)$ = Henry's function. Note that a negative zeta potential signifies a negative net charge, while a positive zeta potential indicates a positive net charge.

The parameter κ, known as the Debye length, is expressed in reciprocal length units. κ^{-1} can be seen as the "thickness" of the electrical double layer. The term 'a' denotes the particle's radius, and κa quantifies the ratio of the particle's radius to the thickness of the electrical double layer. When the Debye length is small compared to the radius of the particle, f(κa) = 1.5 (Smoluchowski model). Meanwhile, if the particle radius is small compared to the Debye length, f(κa) = 1 (Huckel model). Henry's function is valid for low zeta potentials and other models exist for medium and high zeta potentials (Ohshima, 2006).

6.12.2. PROCEDURE

The detailed procedure applied at TU Delft can be seen in Appendix O.

6.12.3. SOURCES OF ERROR

- Due to Kaumera's viscous nature, it is possible that it will stick to the walls of the tubes and the capillary cell. Thus, the system must be cleaned thoroughly between samples.
- Since the capillary tubes are very narrow, this can cause significant shearing while introducing Kaumera, potentially impacting its structure.

6.12.4. ADDITIONAL CONSIDERATIONS

- When testing Kaumera samples (very diluted solutions), use the Smoluchowski model.
- Recommended voltage is between 40-100 Volts. Too high voltages can lead to unwanted convection in the cell (heat production).
- A noticeable reduction in flocculation efficiency has been observed as the Kaumera alkaline solution ages due to hydrolysis. This factor should be taken into account when making comparisons between different Kaumera samples.

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7. ON-SITE CHARACTERIZATION METHODS

7.1. PH

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7.1.1. INTRODUCTION

A pH meter is a tool that measures the acidity and alkalinity of solutions, playing a crucial role in both laboratory settings and fieldwork. In the context of characterization tests, accurately measuring and documenting the pH of a Kaumera sample is imperative to enable meaningful comparisons across samples. The pH level significantly influences the properties of Kaumera, as originally it is an acidic gel but can dissolve effectively under elevated pH conditions. Moreover, during the production of Kaumera, stringent pH control measures are implemented to ensure the consistent delivery of a high-quality product.

7.1.2. PROCEDURE

A detailed procedure on how to clean and calibrate a pH probe and measure pH can be seen in Appendix P.

7.1.3. SOURCES OF ERROR

- When attempting to measure the pH of undiluted Kaumera it is not uncommon to encounter a situation where the pH reading does not stabilize for a reasonable duration. This issue arises primarily because the pH electrode employed may not be suitable for measuring highly viscous samples.
- Measuring pH in protein-containing samples can also be challenging as protein can foul both the pH sensing glass and the ceramic junction, introducing measurement error (Mettler-Toledo International Inc., 2022). As a result, cleaning the pH probe is necessary.

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7.2. CONDUCTIVITY

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7.2.1. INTRODUCTION

A conductivity meter is used to measure the conductivity of solutions, which is a measure of a substance's ability to conduct an electric current. This ability depends on the presence of ions, their concentration, mobility, and valence, as well as temperature (APHA, 1999). Increased salinity leads to higher conductivity since dissolved salts give rise to more ions in the system. Both inorganic (dissolved salts) and organic charged species facilitate conductivity. Conversely, neutral (frequently organic) soluble compounds provide limited ability to conduct electrical current.

Conductivity plays a crucial role in various characterization tests such as acid-base and conductometric titration curves and rheology. Variations in the conductivity of Kaumera can occur due to differences in influent wastewater composition (seawater, industrial, municipal) or the chemicals used during the polymer extraction and precipitation process. For instance, using sodium hydroxide (NaOH) and hydrochloric acid (HCl) may result in an increased presence of sodium chloride and consequently a high-value conductivity (STOWA, 2019).

Higher conductivity values can indicate a greater salt content, which may classify the Kaumera as 'less pure'. However, employing sample preparation methods such as washing (Section 5.1.) or dialysis (Section 5.2.) can effectively reduce Kaumera conductivity. Since the Kaumera polymer itself is charged, it will always contribute to the ionic conductivity, itself or via the counterions associated with the polymer.

It is important to highlight that conductivity is temperature-dependent. To facilitate meaningful comparisons, conductivity measurements should be reported at a standard temperature of 25°C (EPA, 1982). If this is not possible, temperature corrections should be made (see Appendix Q). Conductivity measurements are typically reported in milliSiemens per centimeter (mS/cm), but milliSiemens per meter (mS/m), Siemens per meter (S/m), and microSiemens per cm (µS/cm) are also used. Note: 1 milliSiemens/centimeter (mS/cm) = 100 milliSiemens/meter (mS/m), 1 µmho/cm = 0.1 mS/cm. Once established, conductivity can serve as a reference point for comparing different Kaumera samples.

7.2.2. PROCEDURE

The detailed procedure on how to measure conductivity can be seen in Appendix Q. This protocol was obtained from "Method 120.1: Conductance (Specific Conductance, µmhos 25°C) by Conductivity Meter." (1982) by the United States Environmental Protection Agency [EPA].

7.2.3. SOURCES OF ERROR

- The largest source of potential error are temperature variations and corrections.
- Other problems with conductivity measurements are related to electrode fouling and to inadequate sample circulation.

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7.3. YIELD STRESS: VISCOMETER

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7.3.1. DEFINITIONS

"Yield stress" is defined as minimum stress that must be applied to a sample before it starts to flow (Lee et al., 2015).

7.3.2. INTRODUCTION

In the field, determining Kaumera's yield stress provides operators with an important "product dimension". Yield stress provides insights into the thickness or consistency of the Kaumera gel. In the past, distinguishing whether the produced Kaumera had a more liquid or gel-like nature was a challenging task. Consequently, measuring yield stress became an essential means of comparing different Kaumera samples based on this characteristic. Additionally, measuring yield stress holds significant value in predicting its ability to flow, performance in processes like dipping or coating, and its ease of handling, processing, or utilization.

In the Kaumera pilots, an in-house method is employed to measure yield stress using the Brookfield DV2T Viscometer, providing a quick and easy approach. Comparisons across different samples require the use of the same model instrument, spindle, and protocol. This method yields purely qualitative results, offering relative measurements that are not directly comparable to the quantitative data obtained with a rheometer (refer to Section 6.9.).

7.3.3. METHOD DESCRIPTION

There is no sample preparation method. This implies that samples will have different TS%, so it is important to report this value along with the yield stress.

The process begins by immersing the spindle into the Kaumera sample and rotating it at its maximum speed of 200 rpm for one minute, which effectively disrupts the gel structure. Subsequently, the spindle comes to a halt for 30 seconds, allowing the gel to recover. Following this brief pause, the measurement procedure begins. Initially, the spindle moves at a slow rate of 0.1 rpm, and owing to the resistance posed by the gel, its motion is minimal, resulting in pressure buildup. As the gel eventually breaks, the spindle begins to move, and at this point the yield stress is measured and recorded.

7.3.4. PROCEDURE

The detailed protocol of how to measure yield stress on-site can be seen in Appendix R.

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7.4. YIELD STRESS: LOW-TECH TECHNOLOGIES

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The idea behind low-tech technologies is to promote yield stress measurements on site/during extraction without the use of a rheometer. So far, the following strategies have been tested:

a) The measurement of yield stresses using a funnel, like the slump test for concrete and soil samples: although the slump test for concrete is an empirical test devised to assess the workability and consistency across multiple samples, a more scientifically devised test (using a funnel) can be used to measure the yield stress of Kaumera samples. Upon filling the funnel, the fluid flows through the exit because of the force due to gravity (Fg). The magnitude of this force is proportional to the mass of the sample present within the funnel. Below a certain critical height, the mass of the fluid is not sufficient to sustain the flow as the Fg is balanced by the yield stress. Such measurements provide a quantitative measure of the dynamic yield stress within the sample as opposed to the static yield stress, i.e. the natural stress below which the sample stops flowing.
b) The measurement of yield stresses using plate dipping: the plate dipping method can be used to measure the static yield stress within the sample. In this method, a roughened glass slide is immersed into a beaker of Kaumera and allowed to rest for a fixed period. The resting time is dependent on the time needed for the sample to recover its yield stress. Once recovered, the plate is pulled out in a controlled fashion. The thickness of sample adhered to the surface of the plate is indicative of the yield stress of the sample.
c) The measurement of yield stresses using capillary tubes: like the funnel test, the static yield stress is evaluated by making use of the force exerted due to gravity. Depending on both the diameter of the capillary tube, as well as the angle made by the tube with respect to the vertical axis, a plug flow may be initiated within the capillary tube. These values (diameter and the angle) may then be used to calculate the yield stress within the sample.

Detailed reports about these “low-tech technologies” have been documented in collaboration with students from TU Delft and Hogeschool Utrecht.

8. CONCLUSIONS

In anticipation of Kaumera's open commercialization, standardization emerges as a necessary step to facilitate a cohesive and accelerated advancement in the development of Kaumera extraction technology, characterization, and product development. The compilation of all protocols in a single document not only eases accessibility for all stakeholders but also streamlines the tracking of Kaumera's progress, providing a centralized resource for information that was previously scattered.

Recognizing Kaumera's rapid progressing research and development work, this document is designed to be a 'living' document, regularly updated to incorporate new advances and emerging characterization methods. This ensures that it remains a reliable reference for operators, researchers, and other stakeholders. In addition, when extracting and/or testing Kaumera, it is encouraged to fill in the 'Kaumera sample form' found in Appendix A. Using it will help keep track of the origin and the methods applied to the sample and will later facilitate result comparisons.

In essence, this report not only consolidates the current state of Kaumera research but also paves the way for ongoing advancements and a shared understanding among all stakeholders engaged in its exploration and potential commercialization.

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APPENDICES

APPENDIX A

Kaumera Sample Form

Included in this appendix is the 'Kaumera Sample Form,' which should be filled based on the specific Kaumera sample under analysis. The purpose is to document the sample's origin and characteristics, the procedural steps followed for extraction, and any sample preparation method applied before the characterization methods. This is intended to facilitate easier comparison of results and maintain a clear record of procedures undertaken.

KAUMERA SAMPLE FORM		
1. SLUDGE COLLECTION	Lab ☐	Full-scale ☐
Date:		
Location:		
Comments:		
2. SLUDGE MATERIAL	Granules ☐	Flocs ☐ Combination ☐
Comments (sieved, size distribution, etc.):		
3. KAUMERA EXTRACTION		
Procedure Steps	**Followed**	**Comments/Modifications**
1. Sludge concentration of 5% TS	☐	
2. Water bath to 80 °C	☐	
3. Added 25%(w/v) KOH	☐	
4. Reached pH 10[a]	☐	
5. Stirred thoroughly for 2 hours at 80°C	☐	
6. Centrifuged at 4,000 x g and 30°C for 20 min	☐	
7. Stirred thoroughly while adding 30 wt% HCl (room T°)	☐	
8. Reached final pH of 2.2[b]	☐	
9. Centrifuged at 4,000 x g and 30°C for 20 min	☐	
4. SAMPLE PREPARATION METHOD		
WASHING ☐		
Purpose:		
Washing solution (pH, temperature, ionic strength, composition):		
Repetition of cycles:		
DIALYSIS ☐		
Purpose:		
Dialysate:		
MWCO (kDa):		
Duration:		
DRYING ☐		
Purpose:		
Freeze drying ☐	Other ☐ Specify:	

[a] For optimal product quality this pH might be different and needs adaption, usually within the range of pH 9-11.

[b] For optimal product quality this pH might be different and needs adaption, usually within the range of pH 2-4.

Figure A.1: Kaumera sample form.

APPENDIX B

Lab Extraction Protocol

This appendix includes the standard lab extraction protocol obtained from Bahgat et al. (2023). If adjustments are made to attain a specific goal, it is important to record these modifications. Nevertheless, it is essential to implement any changes thoughtfully and after thorough consideration. Subsequent extractions may be optimized based on the intended end-use application.

MATERIALS

- Excess thickened aerobic granular sludge (5% TS)
- 25% (w/v) KOH
- 30 wt% HCl
- Flask
- Borosilicate glass beaker(s)
- pH sensor
- Magnetic stirring and heating plate (with thermometer)
- Aluminum foil
- Centrifugation tubes
- Centrifuge

EXPERIMENTAL SETUP

In accordance with step (b) outlined in the section Procedure (below), the sludge is placed within a water bath to a temperature of 80°C, as illustrated in Figure B.1. The sludge is contained within a flask, which is then positioned within a 5-liter borosilicate glass beaker designed to withstand elevated temperatures. To retain the heat, insulating material, such as aluminum foil, envelops the beaker. Additionally, the setup incorporates a thermometer and a pH sensor to monitor the temperature and pH levels during the introduction of both the base and acid.

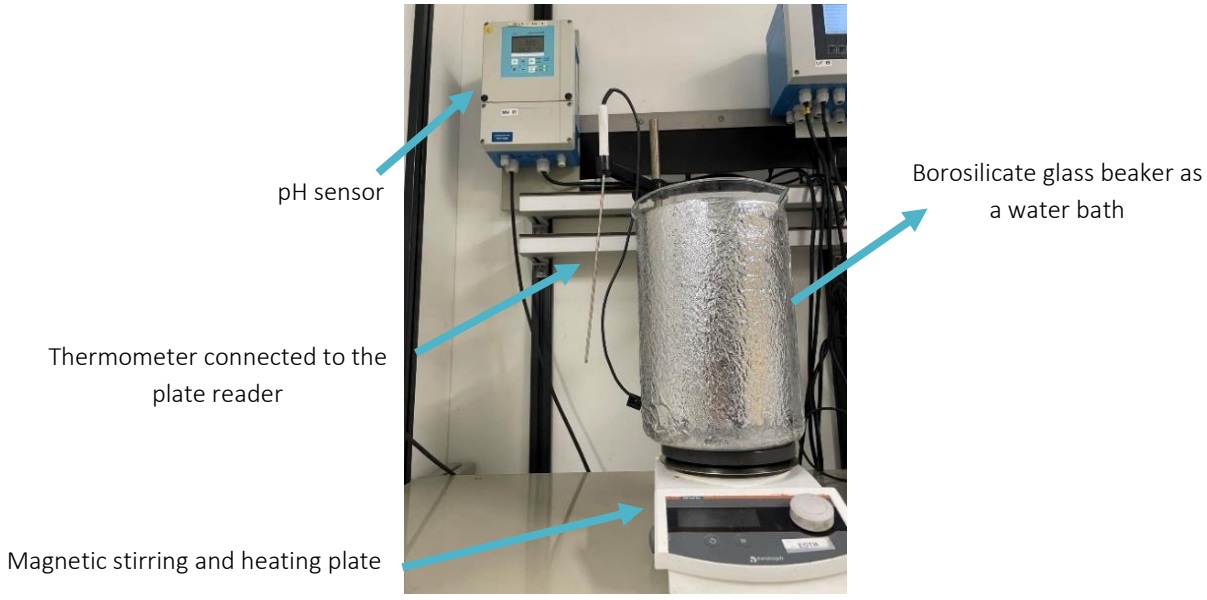


Figure B.1: Water bath setup for Kaumera extraction.

ADDITIONAL CONSIDERATIONS

Selection of the pH probe is critical, as the pH is measured at 80°C. The calibration is either: 1) a probe with ATC and the probe is calibrated using buffers at room temperature, 2) a probe with no ATC and the probe is calibrated using buffers at 80°C. As an example, the Endress+Hauser pH ATC probe with a pH range: of 0 to 14, and a process temperature: of 0 to 135 °C can be used. So, during calibration, ATC reads the true temperature of the buffer and assigns the exact correct value and during testing, ATC reads the true temperature of the sample and adjusts the slope to remain in collaboration. So, the accuracy of the probe is maintained regardless of the temperature. See Appendix P for calibrating the pH probe.

PROCEDURE

Note: no sample preparation method is required for the excess thickened aerobic granular sludge.

a) Collect excess thickened aerobic granular sludge samples (5% TS).
b) Heat weighted amount of fresh excess thickened sludge in a water bath to 80°C.
c) Add 25% (w/v) KOH to the heated sludge until pH 10 is reached (note: for optimal product quality this pH might be different and needs adaption, usually within the range of pH 9-11).
d) Cover the flask and the beaker separately with aluminum foil to prevent evaporation.
e) Stir the mixture for two hours at 80 °C. Ensure thorough mixing to maintain a continuous flow of the liquid, preventing the formation of any stagnant areas or 'dead zones.'
f) Let it cool down to room temperature by putting the flask inside another beaker filled with tap water, leaving the flask open.
g) Transfer the mixture into centrifugation tubes, and centrifuge at 4,000 × g and 30 °C for 20 min.
h) Collect the alkaline sludge residue for analysis.
i) Transfer the alkaline supernatant extract to a glass beaker and stir it slowly at room temperature while adding 30 wt% HCl until it reaches a final pH of 2.2 (note: for optimal product quality this pH might be different and needs adaption, usually within the range of pH 2-4). Mixing should be sufficient to keep the full liquid moving without any ‘dead zones’.
j) Transfer the acidified extract into centrifugation tubes, and centrifuge at 4,000 x g and 30°C for 20 min.
k) Collect the acidic supernatant for analysis and collect the gel-like EPS pellet.

APPENDIX C

Washing

This appendix includes an example of a washing method using HCl solution. This technique enables the removal of both organic molecules and counter ions from the supernatant in an unselective manner, leading to a more uniform composition. This is achieved by exchanging the entire liquid phase with a precisely defined HCl solution.

MATERIALS

- Kaumera sample
- HCl solution
- Electrical Conductivity (EC) meter
- pH probe
- Centrifuge
- Centrifuge tubes

PROCEDURE

a) Measure the pH and conductivity of Kaumera at 25°C as described in Section 7.1. and 7.2. respectively.
b) Prepare an HCl solution with the same pH level as the Kaumera sample. This is done to ensure that the charge of the polymer does not change during washing. Note: it is important to highlight that no salts are introduced at this stage.
c) Roughly dilute the Kaumera gel suspension by a factor of two using the acid solution. Mix thoroughly to promote the dilution of free ions and organic molecules and homogenization of the suspension.
d) Run in the centrifuge at 4000 x g for 10 minutes. If needed, the centrifugation time can be extended to 20 minutes, especially if the total solids percentage (TS%) is significantly high, to achieve separation. However, it is important to maintain the same centrifugation time when comparing different Kaumera samples. Note that the rotor has to be balanced by having containers of the same weight on all sides.
e) Pour out the supernatant. Again, dilute using acid solution by a factor of two and mix thoroughly. Measure the conductivity and pH of the sample.
f) Repeat steps (d) and (e) until a steady (plateau) value is obtained for the conductivity. Small drifts in pH are possible because of the washing away of small molecules.
g) The EC of the samples are generally in the range of 1-2 mS/cm after washing.

ADDITIONAL CONSIDERATIONS

- There exists the risk of losing the polymer if there are changes in pH, conductivity, dilutions, and temperature. It is important to consider this when selecting the appropriate washing solution.
- To achieve a stable conductivity value, it is necessary to repeat steps (d) and (e) up to a maximum of 3-4 times. Each repetition reduces the conductivity by a factor of two.
- If after washing there exists the need to increase the conductivity, it can be brought to the desired value using monovalent salts such as NaCl or KCl. The choice of salt is dependent on the base used for extraction.

APPENDIX D

Dialysis

The method outlined in this appendix is a conventional dialysis procedure implemented at TU Delft to purify EPS samples in a selective manner. Its objective is to eliminate salts and small, freely moving molecular compounds found in the liquid phase (supernatant), while retaining the polymeric substances.

MATERIALS

- Kaumera sample
- 3.5 kDa MWCO dialysis tubing
- Dialysis tubing clips
- Beaker
- Magnetic stirring plate
- *Recommended*: electrical conductivity (EC) meter

PROCEDURE

a) Cut a reasonable amount of dialysis tubing.
b) From one side, fold the corners. Proceed to fold the side two times and seal tightly with a tubing clip.
c) Place a considerable amount of Kaumera sample (approx. 20 ml), making sure to leave some free space on the tubing for it to float when placed in the dialysate.
d) Seal the other side similar to step (b).
e) Fill a two-liter beaker with Milli-Q water.
f) Place the dialysis tube with the sample inside the beaker, making sure it floats (see Figure D.1).
g) Allow it to stir with a magnetic stirrer at a medium level speed.
h) The Milli-Q water must be changed 3-4 times. Each time, for approximately 4 hours. For practical reasons, it can also be left overnight.
i) *Recommended*: the conductivity can be checked with an electrical conductivity (EC) meter to observe if equilibrium has been reached.

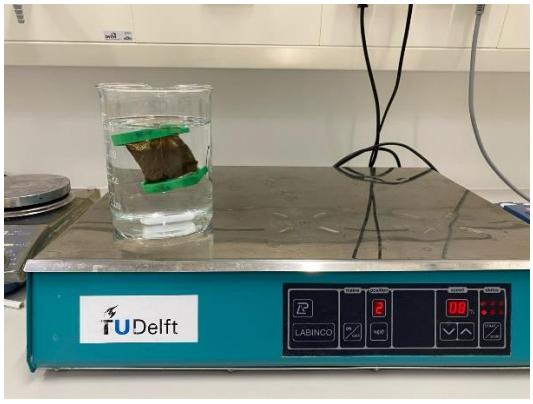


Figure D.1: Dialysis setup with Kaumera sample.

APPENDIX E

Drying: Freeze Drying

This appendix details the process of freeze-drying a Kaumera sample, effectively reducing its moisture content. This method proves advantageous when the sample requires re-dissolution.

MATERIALS

- Kaumera gel sample
- -80°C freezer
- Freeze drier
- High-surface plastic container
- Parafilm
- *Recommended*: Styrofoam box

PROCEDURE

a) Place the sample in a high-surface plastic container.
b) Cap the container with parafilm and close it tightly. With a sharp object, carefully puncture small holes in the parafilm.
c) Place the container with the sample in a freezer at -80°C. Placing the container at an angle of 45° can help increase surface area. The time duration depends on the sample volume and surface area of the container. Generally, the time duration ranges from 30 minutes to 3 hours.
d) Make a visual inspection that the sample is completely frozen. If not, the sample will boil vigorously in the freeze-drier.
e) *Recommended*: when transporting the sample, put it in a Styrofoam box to prevent melting.
f) Place the sample in the freeze-drier. Depending on the sample water content and the surface-to-volume ratio, this might require overnight or even a week of freeze-drying. In general, for small samples of <5 ml, overnight is sufficient. Bigger samples of >5 ml might take a few days or up to a week. Follow the instructions on the manual user guide for correct usage of the freeze-drier.
g) Do a visual inspection to verify the freeze-drying was successful.

APPENDIX F

Volatile Solids (VS) and Total Solids (TS)

This appendix describes how to measure Volatile Solids (VS) and Total Solids (TS) of Kaumera samples according to the procedure outlined in "Standard Methods for the Examination of Water and Wastewater" (1999) by the American Public Health Association (APHA). Perform the measurements with triplicates.

MATERIALS

- Kaumera samples
- Evaporating dishes (e.g., aluminum cups, ceramic or porcelain crucibles)
- Drying oven for operation at 105°C
- Muffle furnace for operation at 550°C
- Desiccator with a desiccant containing a color indicator of moisture
- Analytical balance, capable of weighing up to 0.1 mg

MATERIALS PREPARATION

- Preparation of evaporating dishes: if volatile solids are to be measured, place the clean evaporating dishes in a muffle furnace and ignite at 550°C for one hour. If only totals solids are to be measured, heat evaporating dishes to 105°C for one hour. Store and cool dishes in desiccator for 15 minutes.
- For each sample, choose a volume that will yield a residue between 2.5 and 200 mg.

SAFETY PRECAUTIONS

Since the muffle furnace is extremely hot, be careful when removing the samples from the furnace. Use gloves or tongs if necessary.

PROCEDURE

a) Weigh the dishes, this corresponds to the empty weight (EW).
b) Add the Kaumera samples in the prepared evaporating dishes. Weigh again to obtain the wet weight (WW).
c) Place the samples into the 105°C oven to dry overnight.
d) Take out the samples from the oven and cool in the desiccator for 15 minutes. Weigh to obtain the dry weight (DW).
e) Transfer the samples to a muffle furnace at 550°C for two hours (excluding warming up phase). Due to fire retardant properties of Kaumera, this time should not be shorter.
f) Take out the samples and place in the desiccator for 15 minutes. Weigh to obtain the ash (A).

CALCULATIONS

To obtain TS (g), TS(%), VS (g), and VS(%) use equations F.1, F.2, F.3, F.4, respectively.

$$TS = DW - EW \qquad \text{(F.1)}$$

$$TS\% = \frac{DW-EW}{WW-EW} \qquad \text{(F.2)}$$

$$VS = DW - A \qquad \text{(F.3)}$$

$$VS\% = \frac{DW-A}{DW-EW} \qquad \text{(F.4)}$$

SOURCES OF ERROR

- Always pre-ash the evaporating dish prior to taking measurements. If volatile solids are to be measured, ignite clean evaporating dish at 550°C for one hour in a muffle furnace. If only total solids are to be measured, heat the clean dish to 105°C for one hour. This is to ensure residual material from the production of the dishes are completely removed and is not taken into account as part of the ash weight.
- Make all weighing quickly as wet samples lose weight by evaporation. After drying or ignition, residues are also hygroscopic and can rapidly absorb moisture from the air.
- When a portion of the sample adheres to the surface, it is important to take this into account when assessing and documenting the results. In certain cases, samples may form a crust during drying, which can impede water evaporation.
- Reduce the frequency of opening the desiccator as it allows humid air to enter.
- Residues dried at 105°C can retain crystallized water and mechanically occluded water.
- It is important to note that due to the fire-retardant characteristics of Kaumera, there exists the risk that complete combustion may not have occurred after the established two hours. For more precise and accurate results, it is an option to extend the samples' exposure in the oven until the weight change is less than 4%.
- Ceramic crucibles are preferred sample holders over aluminum cups, as the quality of the aluminum cup can differ per manufacturer and influence the measurement.

APPENDIX G

BCA Assay for Total Protein Determination

This appendix outlines the procedure for preparing Kaumera samples before conducting the BCA assay for the measurement of total proteins.

SAMPLE PREPARATION

To begin, the acidic gel sample is dialyzed using a 3.5 kDa dialysis membrane against Milli-Q water (as detailed in Section 5.2.). This step is applied for removing salts, as a high concentration of salt could hinder the biuret reaction. Eliminating salts also simplifies the comparison of samples that have undergone different extraction procedures.

Following the dialysis process, the samples are freeze-dried according to the procedure outlined in Section 5.3. Subsequently, the dried Kaumera is dissolved in an alkaline solution prepared with Milli-Q water. Sodium hydroxide solution is a common choice, but alternative strong bases can be employed. However, it is important to ensure that the specific cation used does not disrupt the analysis and does not introduce unnecessary variables.

Furthermore, the concentration of NaOH solution required depends on the EPS being analyzed. In the past, concentrations of 0.01 M NaOH or 0.02 M NaOH (Felz et al., 2019) have been employed. However, if the EPS is not dissolving properly, a concentration of 0.1 M NaOH may be used. It is advisable to experiment with different concentrations to determine which one allows for the complete dissolution of the EPS.

Once a known concentration of EPS (e.g., 1000 mg/L) is dissolved in the alkaline solution, it is stirred overnight at a moderate speed. If there are difficulties in dissolving the EPS, it is possible to apply gentle heat while stirring. However, a low temperature (around 50°C or even lower) should be used since proteins already denature at 80°C. When the EPS is fully dissolved, it should appear clear without any turbidity.

In the comparison of research studies, the use of different concentrations or alkaline solutions does not pose limitations as long as the EPS is fully dissolved, and the standards are also dissolved in the same alkaline solution as the EPS.

PROCEDURE

The detailed step-by-step procedure for performing the BCA assay can be found here:

"Pierce™ BCA Protein Assay Kit (Catalog Numbers 23225 or 23227)". Thermo Fisher. https://www.thermofisher.com/order/catalog/product/es/es/23225. Published January 30, 2020.

APPENDIX H

GC-MS for Amino Acid Analysis

This appendix outlines the sample and standard preparation for GC-MS as well as the step-by-step procedure.

SAFETY PRECAUTIONS

For the derivatization, work in the fume hood and wear nitrile gloves.

SAMPLE PREPARATION

If the goal is to quantify the *total amino acids* (Felz et al., 2020):

a) Conduct acid hydrolysis on a sample with a concentration of 2 mg/ml in 6 M hydrochloric acid at 105°C for 24 hours.
b) Neutralize the sample with 6 M sodium hydroxide and centrifuge at 10,000 x g for 5 minutes.
c) Filter the supernatant with a 0.45 µm PVDF filter and dilute 1:5 with ultrapure water.
d) Pipet 100 µl of sample into a GC-vial.
e) Add 30 µl of (100mgNaCl + 1ml Milli-Q) to each vial. Note: the added salts serve as dehydrating agents, particularly during the subsequent freeze-drying process.
f) Add 20 µL of an internal amino acid standard (labeled biomass, ^{13}C extract; Wahl et al., 2014) to each vial. Note: When selecting an appropriate internal standard, it is crucial to ensure that it is absent from the sample matrix and does not interfere with other compounds present. Ideally, the chosen compound should closely resemble the target analyte in nature, exhibiting similar characteristics such as retention time, peak shape, and response.
g) Close the vials with the caps containing white Teflon septa.
h) Make 2 holes into the Teflon septa.
i) Freeze dry your samples as described in Section 5.3.
j) Recap the samples with the caps with natural rubber/PTFE septa.
k) Switch on the heating block (70°C).
l) Add 75µl Acetonitrile and 75µl of MTBSTFA + 1% TBDMCS Silylation Reagent (Thermo Scientific™, catalog number: TS-48920) (at room temperature) to each vial.
m) Place the samples for 60 minutes in the heating block at 70°C.
n) Let them cool down to room temperature.
o) Transfer the samples into the glass tubes for centrifugation.
p) Centrifuge for 2 minutes at 10,000 x g.
q) Transfer 80µl of the supernatant back into the original GC-vial and remove air bubbles.

To quantify the *free amino acids* in the liquid phase (supernatant):

a) Centrifuge the Kaumera for 15 minutes at 8671 x g. Collect supernatant.
b) Use a spin column (Vivaspin® 6, 5,000 MWCO PES, 25pc) and collect the permeate.
c) Pipet 100 µl of sample into a GC-vial.
d) Add 30 µl of (100mgNaCl + 1ml Milli-Q) to each vial. Note: the added salts serve as dehydrating agents, particularly during the subsequent freeze-drying process.
e) Add 20 µL of an internal amino acid standard (labeled biomass, ^{13}C extract; Wahl et al., 2014) to each vial. Note: When selecting an appropriate internal standard, it is crucial to ensure that it is absent from the sample

matrix and does not interfere with other compounds present. Ideally, the chosen compound should closely resemble the target analyte in nature, exhibiting similar characteristics such as retention time, peak shape, and response.

f) Close the vials with the caps containing white Teflon septa.
g) Make 2 holes into the Teflon septa before freezing drying.
h) Freeze dry your sample as described in Section 5.3.
i) Recap the samples with the caps with natural rubber/PTFE septa.
j) Switch on the heating block (70°C).
k) Add 75µl Acetonitrile and 75µl of MTBSTFA + 1% TBDMCS Silylation Reagent (Thermo Scientific™, catalog number: TS-48920) (at room temperature) to each vial.
l) Place the samples for 60 minutes in the heating block at 70°C.
m) Let them cool down to room temperature.
n) Transfer the sample into the glass tubes for centrifugation.
o) Centrifuge for 2 minutes at 10,000 x g.
p) Transfer 80µl of the supernatant back into the original GC-vial and remove air bubbles.

STANDARDS PREPARATION

Use the concentration of standards found in Table H.1. It is important to understand that the standards follow the same procedures as the samples. This involves adding the same quantities of salt solution (30µl of [100mg NaCl + 1ml Milli-Q]) and 20 µl of internal standard to each vial. Subsequently, the standards also undergo freeze-drying, derivatization, and centrifugation.

Table H.1: Amino acid concentrations for standards.

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	
Ala	2.19	4.38	10.94	21.88	43.75	87.51	131.26	218.77	437.53	875.07	µM
Pyr	0.20	0.40	1.01	2.01	4.02	8.05	12.07	20.11	40.23	80.45	µM
Gly	0.28	0.57	1.42	2.84	5.68	11.36	17.04	28.40	56.80	113.60	µM
Val	0.51	1.01	2.53	5.06	10.12	20.25	30.37	50.62	101.24	202.48	µM
Leu	0.06	0.12	0.30	0.60	1.20	2.40	3.60	6.00	12.00	24.00	µM
Ile	0.06	0.12	0.31	0.61	1.22	2.44	3.66	6.11	12.21	24.43	µM
Pro	0.18	0.37	0.91	1.83	3.65	7.30	10.95	18.25	36.50	73.01	µM
Ser	0.51	1.02	2.54	5.09	10.17	20.35	30.52	50.86	101.73	203.45	µM
Thr	0.51	1.01	2.54	5.07	10.15	20.29	30.44	50.73	101.46	202.91	µM
Met	0.06	0.12	0.29	0.59	1.17	2.34	3.51	5.86	11.71	23.42	µM
Asp	1.74	3.48	8.69	17.39	34.78	69.56	104.33	173.89	347.78	695.57	µM
Phe	0.04	0.08	0.20	0.40	0.80	1.61	2.41	4.02	8.04	16.08	µM
Cys	0.06	0.12	0.30	0.60	1.19	2.38	3.57	5.95	11.91	23.81	µM
Orn	0.23	0.47	1.17	2.33	4.67	9.33	14.00	23.34	46.67	93.35	µM
Glu	3.97	7.94	19.85	39.70	79.39	158.79	238.18	396.97	793.94	1587.88	µM
Lys	0.24	0.48	1.19	2.38	4.76	9.52	14.28	23.81	47.62	95.23	µM
Asn	0.25	0.49	1.23	2.46	4.91	9.83	14.74	24.57	49.14	98.28	µM
AAA	0.19	0.37	0.93	1.87	3.73	7.47	11.20	18.66	37.33	74.65	µM
Gln	2.03	4.06	10.14	20.28	40.56	81.12	121.68	202.79	405.59	811.18	µM
Tyr	0.06	0.12	0.30	0.59	1.18	2.37	3.55	5.92	11.85	23.69	µM
His	0.24	0.48	1.20	2.40	4.81	9.62	14.42	24.04	48.08	96.16	µM
Trp	0.05	0.10	0.25	0.50	0.99	1.99	2.98	4.97	9.94	19.89	µM

PROCEDURE

As described in Felz et al. (2020):

a) 80 µL of each sample are introduced into the GC-MS (7890A GC, Agilent) together with a 5975C single quadrupole mass spectrometer (Agilent) (de Jonge et al., 2011).

b) Out of the 80 µL, 1 µL is injected on a Zebron ZB-50 column (30 m x 250 µm internal diameter, 0.25 µm film thickness; Phenomenex, Torrance, CA, USA) for injection in splitless mode by a programmed temperature vaporizer (PTV; Gerstel, Mühlheim, Germany). Straight glass liners with glass wool are used (Agilent).

c) MS is operated in selected ion monitoring mode and the quantification of amino acids performed by isotope dilution mass spectrometry.

ADDITIONAL CONSIDERATIONS

- High levels of phosphates and sulfates in the sample can deplete the derivatizing reagent and affect chromatography, leading to distorted peak shapes and diminished responses.
- For research comparisons, it is advisable to compare the relative retention time between amino acids to see if they are similar to each other.
- The GC-MS system should be fine-tuned to prevent any overlap or superimposition of chromatographic peaks when two or more compounds share the same retention time.

APPENDIX I

Phenol-Sulfuric Acid Method for Total Carbohydrate Determination

This appendix includes the procedure for determining the total carbohydrates of a Kaumera sample in equivalents of the used sugar standard (glucose). This protocol was adjusted from: Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. T., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. Analytical chemistry, 28(3), 350-356.

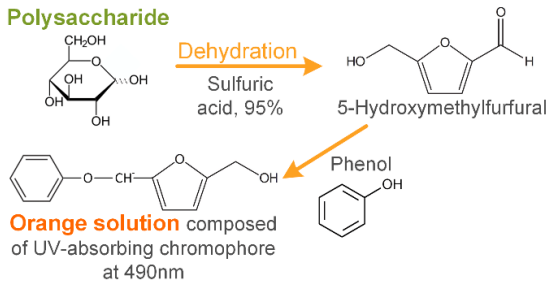


Figure I.1: Graphic representation of the phenol-sulfuric acid method.

SAMPLE PREPARATION

To initiate the sample preparation, the acidic gel sample is subjected to dialysis, employing a 3.5 kDa dialysis membrane, against Milli-Q water, as outlined in Section 5.2. This step serves the purpose of salt removal. In the phenol-sulfuric acid method, the presence of high salt concentrations can impede reactions and influence the outcomes. Eliminating salts also simplifies the comparison of samples that have undergone different extraction procedures.

Following the dialysis process, the samples are freeze-dried according to the procedure outlined in Section 5.3. Subsequently, the dried Kaumera is dissolved in an alkaline solution prepared with Milli-Q water. Sodium hydroxide solution is a common choice, but alternative strong bases can be employed. However, it is important to ensure that the specific cation used does not disrupt the analysis and does not introduce unnecessary variables.

Furthermore, the concentration of NaOH solution required depends on the EPS being analyzed. In the past, concentrations of 0.01 M NaOH or 0.02 M NaOH (Felz et al., 2019) have been employed. However, if the EPS is not dissolving properly, a concentration of 0.1 M NaOH may be used. It is advisable to experiment with different concentrations to determine which one allows for the complete dissolution of the EPS.

Once a known concentration of EPS (e.g., 1000 mg/L) is dissolved in the alkaline solution, it is stirred overnight at a moderate speed. If there are difficulties in dissolving the EPS, it is possible to apply gentle heat while stirring. However, a low temperature (around 50°C or even lower) should be used since high temperatures can affect the EPS structure. When the EPS is fully dissolved, it should appear clear without any turbidity.

In the comparison of research studies, the use of different concentrations or alkaline solutions does not pose limitations as long as the EPS is fully dissolved, and the standards are also dissolved in the same alkaline solution as the EPS.

MATERIALS

- Re-dissolved Kaumera samples in alkaline solution
- 95-97% sulfuric acid (concentrated)
- 5% w/v phenol (5 gram phenol, fill up to 100 ml Milli-Q water)
- Glucose standard of 200 mg/L concentration in Milli-Q water (10 mg in 50 ml)
- Glass borosilicate tubes
- 5 ml plastic pipettes and balloon
- 2.5 ml cuvettes
- Spectrophotometer (D3 3900, HACH)

SAFETY PRECAUTIONS

Work in the fume hood and use goggles and gloves.

PROCEDURE

a) Prepare 1500 µl of the glucose standards with concentrations 0 – 200 mg/L.

Table I.1: Required volumes for glucose standards preparation.

Concentration (mg/L)	200	180	150	120	90	60	30	0
Standard solution (µL)	1500	1350	1125	900	675	450	225	0
Milli-Q water (µL)	0	150	375	600	825	1050	1275	1500

b) Pipette 300 µL of each standard solution and 300 µL of sample in triplicates in the glass borosilicate tubes.
c) Add 300 µL of 5% w/v phenol.
d) Immediately add 1500 µL of sulfuric acid. – Caution when pipetting the sulfuric acid, because it has a high viscosity, it is recommended to reverse pipette. – Carefully vortex sample tubes, since it can be very hot.
e) Incubate at room temperature for 20 min – in the fume hood. – Meanwhile, blank the spectrophotometer with 2 ml Milli-Q water in a cuvette, and place the 5 ml plastic pipettes next to the spectrophotometer.
f) Use a 5 ml plastic pipette and a balloon - Pipet 2000 µL of the solution into a 2.5 ml cuvette. – *Dispose the plastic pipette after each sample and use a new one.*
g) Measure at A_{490} nm (for C6 sugar).
    a. Turn on the spectrophotometer by pressing the button shown with arrow (1) (Figure I.2).

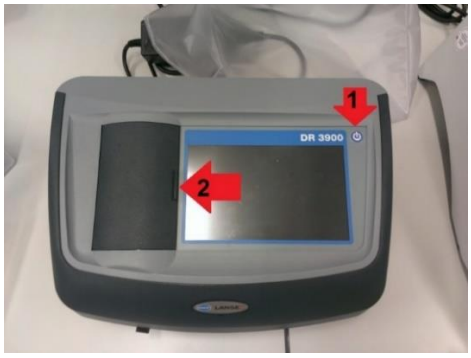


Figure I.2: Spectrophotometer (D3 3900, HACH)

b. Open the chamber depicted with arrow (2) (Figure I.2) on the left to check which measuring device is currently being used.
c. Change (if necessary) from the barcode setup to the cuvette setup.
    i. Remove the plastic part in the light channel (Figure I.3a).
    ii. Add the plastic part for cuvette measurements (Figure I.3b) – the direction of this part is indicated with the arrow (1) (Figure I.3c).
    iii. Introduce the cuvette in the compartment shown with arrow (2) (Figure I.3c) so that the arrow shown on the cuvette (Figure I.3c) is in the direction of the light. Note: only touch the cuvette at the upper part, if accidently touched at the lower part, clean it with a tissue.

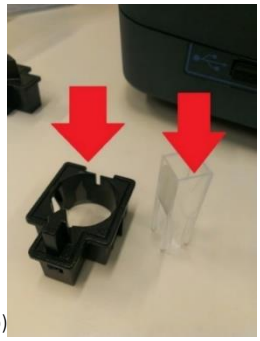
(a)  (b)  (c) 

Figure I.3: Procedure on how to introduce cuvette in the spectrophotometer: (a) remove the plastic part in the light channel, (b) add the plastic part for cuvette measurements, (c) indication of placement direction.

d) Select “Single Wavelength” (Figure I.4).

Figure I.4: Main menu in the spectrophotometer D3 3900 (Hatch).

e) Select “Absorbance” as indicated with arrow (1) (Figure I.5) and adjust the wavelength “**λ**” to 490 nm (C_6 sugar) as indicated with arrow (2) (Figure I.5).

Figure I.5. Menu after "Single Wavelength" selection in the spectrophotometer D3 3900 (Hatch).

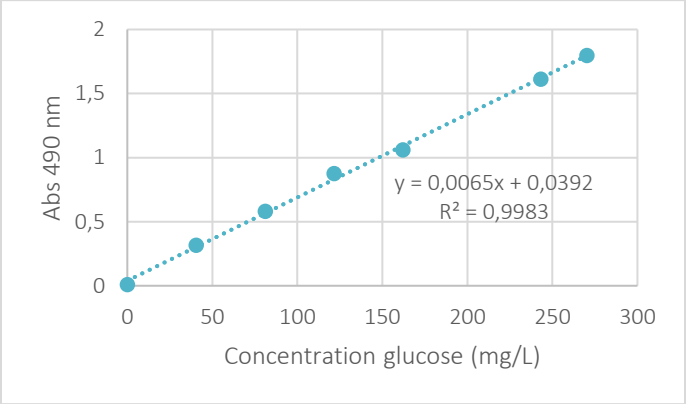


Figure I.6: Example of calibration curve constructed with glucose standards.

APPENDIX J

HPAEC-PAD for Monosaccharide Analysis

In this appendix, a comprehensive guide is provided on sample preparation and the step-by-step procedure for performing an HPAEC-PAD analysis on Kaumera samples. Suggestions on how to overcome common problems with the HPAEC-PAD are also included.

STANDARDS PREPARATION

- Prepare all solutions/dilutions with ultrapure water.
- Prepare stock solutions of the sugar monomers of interest (e.g., glycerol, fucose, galactose, glucose, mannose, rhamnose, ribose, xylose, galactosamine, glucosamine, galacturonic acid, and glucuronic acid) with a concentration of 10 mg/ml.
- *For qualitative analysis*: mix the standard solutions to obtain a standard mix containing 0.01 mg/ml of each sugar of interest.
- *For quantitative analysis*: preparing all the sugars in only two stock solutions allows for a sugar quantification with significantly less dilution series. To obtain a standard line for each sugar:
    - Prepare a standard solution (I) containing 0.25 mg/ml of each of the following sugars: glycerol, galactosamine, glucosamine, glucose, xylose and galacturonic acid. Dilute this solution to obtain solutions with concentrations of 0.01 mg/ml, 0.025 mg/ml, 0.05 mg/ml, 0.1 mg/ml, 0.25 mg/ml of each sugar.
    - Prepare a standard solution (II) containing 0.25 mg/ml of each of the following sugars: fucose, rhamnose, galactose, mannose, ribose and glucuronic acid. Dilute this solution to obtain solutions with concentrations of 0.01 mg/ml, 0.025 mg/ml, 0.05 mg/ml, 0.1 mg/ml, 0.25 mg/ml of each sugar.
- These will be used to obtain a standard line for each sugar.
- Transfer 1 ml of each of the standard solutions into an analysis vial.

SAMPLE PREPARATION

a) A desalting step is required. Dialysis can be done by using a 3.5 kDa MWCO membrane against Milli-Q water (Section 5.2.).
b) Freeze-dry the sample (refer to Section 5.3.).
c) Hydrolyze the dried sample at a concentration of 10 mg/ml in 1 M HCl for 8 hours at 105°C in a test tube with a screw cap. It is important that the test tube is closed tightly.
d) Centrifuge the hydrolyzed sample at 10,000 × g for 5 min and collect the supernatant.
e) Neutralize the supernatant with an equivalent volume of 1 M NaOH.
f) Dilute the neutralized sample 1:5 with Milli-Q water.
g) Filter it through a 0.45 µm PVDF filter. Do not use CAS filters as this will introduce glycerol to the sample.

The hydrolyzed samples contain approximately 200 mM NaCl, which can affect the results. Therefore, spiking the sample with the sugar standards is necessary to certainly identify present monomers.

- *Spiked sample (normal sample + standards):* mix 800 µL of the diluted and filtered sample with 200 µL of the standard mixture containing all sugars at a concentration of 0.01 mg/ml.
- *Normal sample*: Mix another 800 µL of this sample with 200 µL of ultrapure water.
- These two samples will be overlaid after the HPAEC-PAD analysis to visualize the present sugar monomers.

ELUENTS PREPARATION

- Milli-Q water cleaning solution – 2 L
  a) Disconnect bottle from the HPAEC-PAD equipment at < 8 psi
  b) Use residual Milli-Q water in the bottle to clean helium gas sparger and adjust size of the helium bubbles to small bubbles.
  c) Discard the old Milli-Q water
  d) Wash the bottle three times with ~100 ml Milli-Q water
  e) Fill up the bottle with 2 L Milli-Q water
  f) Connect the bottle to the HPAEC-PAD
- Eluent A: 200 mM sodium hydroxide (NaOH) – 2 L
  a) Clean a 1000 ml volumetric flask three times with Milli-Q water
  b) Close the valve of the bottle
  c) Remove the bottle and discard the residual NaOH-solution
  d) Wash the bottle three times with ~100 ml Milli-Q water
  e) Measure 1000 ml Milli-Q water in a volumetric flask – remove 21.2 ml with a pipette. Transfer the water into the eluent bottle. Measure another 1000 ml in a volumetric flask and transfer the water into the eluent bottle. Close the bottle with aluminum foil.
  f) Sparge the water for 7 min with helium gas (small bubbles)
  g) Add 21.2 ml ultrapure 50% (w,w) NaOH solution with a pipette. Note: 50 % (w,w) NaOH water ≙ 18.92 mol/L. Use a clean pipette tip each time.
  h) Add the NaOH directly in the water to avoid introduction of CO_2 into the liquid which would turn into CO_3^- at the present pH (> 12) and falsify the later analysis.
  i) Do not shake the NaOH bottle as by this atmospheric CO_2 could be introduced resulting in Na_2CO_3, which settles on the bottom.
  j) Always take liquid from the center of the bottle not from the bottom (if the bottle is filled less than 30%, check first for impurities).
  k) Connect the bottle to the HPAEC-PAD
  l) Open the valve then wait for 5 min
  m) Shake the bottle thoroughly
- Eluent B: sodium acetate – 50 mM NaOAc, 200 mM NaOH – 2 L (note: If a stronger sodium acetate eluent is needed, prepare eluent B*).
  a) Clean a 1000 ml volumetric flask three times with Milli-Q water
  b) Clean a 500 ml volumetric flask three times with Milli-Q water
  c) Close the valve of the bottle
  d) Remove the bottle and discard the residual NaOH-NaOAc – solution
  e) Wash the bottle three times with ~100 ml Milli-Q water
  f) Measure 1000 ml Milli-Q water in a volumetric flask – remove 21.2 ml with a pipette. Transfer the water into the eluent bottle.
  g) Fill up the 500 ml flask with ~0.3 L Milli-Q water
  h) Add 8.2 g of ultrapure anhydrous NaOAc and mix with magnetic stir bar for ~10 min until all of the NaOAc is dissolved.
  i) Fill up the solution to ~0.5 L with Milli-Q water and mix again shortly
  j) Wash the Schott Duran filter three times with ~150 ml Milli-Q water
  k) Wash the sucking bottle three times with ~150 ml Milli-Q water

l) Filter the NaOAC solution with the Schott Duran filter and transfer the elution into the clean 1000 ml volumetric flask.
m) Fill up the volumetric flask to 1000 ml and transfer the liquid into the elution bottle. Close the bottle with aluminum foil.
n) Sparge for 7 min with helium gas (small bubbles)
o) Add 21.2 ml ultrapure 50% (w,w) NaOH solution with a pipette (handle the same way as described above).
p) Connect the bottle to the HPAEC-PAD
q) Open the valve and wait for 5 min
r) Shake the bottle thoroughly
s) Clean the helium sparger with Milli-Q water to remove NaOAc residues.

- Eluent B*: 500 mM NaOAc, 200 mM NaOH – 0.25 L
  a) Clean a 250 ml volumetric flask with Milli-Q water
  b) Fill up the flask with ~0.15 L Milli-Q water
  c) Add 10.3 g of ultrapure anhydrous NaOAc and mix with magnetic stir bar for ~10 min until all of the NaOAc is dissolved.
  d) Fill up the solution to 0.25 L with Milli-Q water and mix again shortly
- Eluent C and D: Milli-Q water eluent – 2 L
  a) Close the valve of the bottle
  b) Remove the bottle and discard the residual water
  c) Wash the bottle three times with ~100 ml Milli-Q water
  d) Fill up with 2 L Milli-Q water and cover the bottle with aluminum foil
  e) Sparge the water for 7 min with helium gas (small bubbles)
  f) Connect the bottle to the HPAEC-PAD and open the valve

EQUIPMENT

- Dionex ICS-5000+
- AminoTrap pre-column (Dionex)
- CarboPac PA20 column (Dionex)

PROCEDURE

a) Prime the pump. Connect all eluent bottles to the equipment. Open valve of the pump 50%. Chose elution B, C, and D at 25% to achieve priming for all four pumps. During priming, make sure no liquid comes out of the valve. When priming is done, close the valve of the pump.
b) Open the software to be used. Here, Chromeleon™ Chromatography Data System (CDS) Software (catalog number: CHROMELEON7) is used. Create a new sequence and select ICS-5000. Enter the number of samples, the amount of injections per vial, and the start position of your samples (BA1, GA1 or RA1).
c) Choose an instrument (isocratic or gradient) and processing method.
d) How to create a new instrument method (isocratic):
    a. Choose the run time length.
    b. Set column flow at 0.5 ml/min (never exceed this rate) and keep the lower pressure limit at 0 psi and the upper limit at 5000 psi (never above 5000 psi).
    c. Adjust the elution: elution A (NaOH) cannot be changed and is adjusted automatically. For an isocratic method, elution B (or B*) (NaOH + NaOAc) is not needed and set at 0. Elution C and D (Milli-Q water) have to be adjusted depending on the desired NaOH concentration for the elution of the

sample in the column. Elution D is only needed for the mixing if Elution C is depleted. As an example, the NaOH elution in bottle A has a concentration of 200 mM and 5 mM are needed for the elution of the sample compounds. Then adjust 97.5% for bottle C and bottle A will automatically be adjusted to 2.5%.

d. Choose the mode (Integrated Amperometry or DC Amperometry). If available, pick Integrated Amperometry.
e. Turn on the amperometry cell. Select the data collection rate at 2 Hz and turn on autozero. The data collection rate is important for the resolution of the peaks. If very narrow peaks are present a higher value is better, if very broad peaks are present a low value is suitable.
f. Choose a quadruple waveform (e.g., Gold, Ag-AgCl RE, Carbo, Quad). Leave the lower pH limit at 10 and the upper limit at 13.
g. Set the column temperature to 30°C and the compartment temperature to 15°C.

e) How to create a new instrument method (gradient):
   a. Enter the run time length of the isocratic part of the elution.
   b. Choose the type of the method, here pick multi-step gradient (isocratic or gradient).
   c. Enter the names of the elution (e.g., %A 200mM NaOH, %B 200 mM NaOH + 500mM NaOAc, %C and %D Milli-Q water).
   d. Enter 0.5 mL/min as flow rate (maximum flow rate). The first part of the elution is always isocratic, therefore here leave B (NaOH + NaOAc) at 0. Then adjust C to obtain the desired concentration of A (NaOH) during the isocratic part. D can be left at 0 as C and D contain Milli-Q water and only C is needed. Choose a curve of 5 to obtain a fast change of isocratic to gradient method.
   e. Set the starting point of the gradient, so the isocratic method is long enough to elute neutral and amino sugars before the gradient starts (here 13 min). All other values should be the same.
   f. Set-up the gradient. Flow-rate, D and the curve always stay the same. First determine the starting point of the gradient, here 13.1 min and the initial NaOAc percentage, here 4%. Then set the end of the gradient, here 23.1 min and the final percentage of NaOAc, here 40%. All compounds of the sample should be eluted after this amount of NaOAc.
   g. Afterwards apply a washing step of the column of at least 3 min to remove possible residual sample compounds, here 7 min. Then remove all the attached carbonate residues of the column by applying only NaOH (elution A) to the column for 5 min. The time values are based on experience with previous experiments.
   h. After the washing step, the column needs to be stabilized for following analysis, therefore the same conditions as during the isocratic part are applied for 15 min.
   i. Choose the mode (Integrated Amperometry or DC Amperometry). If available, pick Integrated Amperometry.
   j. Turn on the amperometry cell. Select the data collection rate at 2 Hz and turn on autozero. The data collection rate is important for the resolution of the peaks. If very narrow peaks are present a higher value is better, if very broad peaks are present a low value is suitable.
   k. Choose a quadruple waveform (e.g., Gold, Ag-AgCl RE, Carbo, Quad). Leave the lower pH limit at 10 and the upper limit at 13.
   l. Set the column temperature to 30°C and the compartment temperature to 15°C.

f) Analysis:
   a. Performed with a Dionex ICS-5000+, AminoTrap pre-column, PA20 column, ultrapure water, 200 mM NaOH, 200 mM NaOH + 50 mM Na-acetate.
   b. Transfer 1 ml of ultrapure water in an analysis vial.
   c. Transfer 1 ml of each of the standard solutions in an analysis vial.

d. Transfer 1 ml of the spiked sample in an analysis vial and 1 ml of the normal sample in an analysis vial.
e. Transfer 1 ml of each diluted and filtered sample in an analysis vial.
f. Before each first use, wash the column according to Table J.1.
g. Equilibrate the column by injection three ultrapure water samples and run the analysis according to the sample elution in Table J.1. Evaluate that no molecules are eluted from the column and the chromatogram stays the same.
h. Analyze the standards according to Table J.1 with sample elution.
i. Analyze the samples according to the Table J.1 with sample elution.
j. After all samples are analyzed, analyze again one of the sugar standards to evaluate a possible sensitivity decrease of the equipment.
k. After 10-15 samples, perform a column wash again by injecting three Milli-Q water samples and analyze the standard again before injecting new samples to prevent a decrease in sensitivity.

g) Sugar monomers can be qualitatively visualized by overlaying the spiked and the normal sample.
h) Sugar monomers can be quantified with the standard lines. Standard lines need to be prepared every time the analysis is performed.

Table J.1: Column wash and sample elution depending on the desired sugar analysis.

Step	Eluents	Duration	Reason
Column wash	200 mM NaOH + 50 mM Na-acetate	30 min	Removal of strongly bound substances from the column
	200 mM NaOH	90 min	Removal of acetate from the column
	2 mM NaOH	12 mM	Equilibration of the column
Sample elution	2 mM NaOH	30 min	Elution of sugar alcohols, neutral sugars and amino sugars
	200 mM NaOH + 50 mM Na-acetate	15 min	Elution of large neutral sugars and uronic acids
	200 mM NaOH	15 min	Washing of the column
	2 mM NaOH	12 min	Equilibration of the column

SOURCES OF ERROR

- Harsh hydrolytic conditions are needed to obtain sugar monomers. However, different sugar monomers show a different stability towards the hydrolysis conditions (e.g., the concentration of neutral sugars decreases with an extended hydrolysis time, whereas the concentration of amino sugars and glycerol increases).
- N-acetyl-galactosamine/-glucosamine will be de-acetylated during the extraction and hydrolysis conditions and will only be measured as galactosamine and glucosamine.
- Measured glycerol can be a hydrolysis artefact of hydrolyzed sugar monomers.
- Increased concentrations of inorganic anions can decrease the sensitivity of the column.
- Attention should be paid to the preparation of the buffers (eluents) in order to avoid the presence of carbon dioxide and subsequent production of carbonate. Carbonate (divalent ion at pH 12) binds strongly to the anion exchangers and interferes with carbohydrate retention, decreases column selectivity, and loss in resolution.
- Peaks always shift because the column, the sample, or eluents are different.

TROUBLE SHOOTING

1. If the height of the peaks is very low:
    a. If possible, increase the sample concentration.
    b. At higher sodium hydroxide concentrations, the peak height increases (e.g., 10 mM sodium hydroxide results in much higher and sharper peaks than 2 mM sodium hydroxide).
    c. Wash the column (30 min 200 mM NaOH + 50 mM Na-acetate, 90 min 200 mM NaOH, 12 min equilibration). If nothing changes, measure a sugar standard to see if the peak is much lower than normal. If this is the case, a new column is necessary.
2. Peaks are not separating well:
    a. If possible, decrease the sodium hydroxide concentration. At 2 mM sodium hydroxide, there is still good resolution and reproducible results are obtained (peaks will be less sharp and broader than at higher sodium hydroxide concentrations).
3. The sample contains a high concentration of salt:
    a. Wash the column thoroughly with sodium hydroxide and sodium acetate to avoid salt being bounded to the column.
4. The retention time of sugars eluted with acetate is changing with each new sample:
    a. The change of retention time is severe if the entire elution is only performed with sodium acetate/sodium hydroxide. Adding an elution part without sodium acetate decreases the intensity of change of retention time.
    b. Wash the column after a few samples as described under 1.
    c. Alternatively, add a sample spiked with standards every few samples to compensate for the retention time change.
5. Peaks appear which shouldn't appear:
    a. Wash the column thoroughly with sodium hydroxide and sodium acetate to exclude the measurement of compounds which were bound to the column from previous measurements (see under 1).
    b. Check when the eluents were changed for the last time, especially the sodium acetate eluent should be replaced after two weeks.
6. Standards in a pure standard solution have a different retention time than the same standards in the sample:
    a. Spike the sample with the sugar standards and run the sample by itself and spiked to reduce the influence of parameters affecting the retention time such as high salt concentration.
7. The retention time of all peaks is not constant:
    a. Check when the pH reference electrode was changed for the last time. If the problem continues after the washing the column and for neutral sugars at low NaOH concentration, a change of the pH electrode is necessary.
8. The sensitivity is decreasing:
    a. Wash the column as described in 1.
    b. If washing of the column does not help, check the surface of the gold electrode. If the surface is not shiny, clean the surface as described in the manual user guide.

APPENDIX K

Proteins and Carbohydrates: Indirect Measurements

This appendix provides detailed information on how to calculate total protein and carbohydrate content from COD, Total N, ammonium, and VS measurements (Section 6.1.), according to "Chapter 14 - Biochemical Conversion: Anaerobic Digestion" (Kleerebezem, 2014) from "Biomass as a Sustainable Energy Source for the Future: Fundamentals of Conversion Processes" (De Jong & Van Ommen, 2014).

MATERIALS

- Kaumera sample
- Hach Lange COD kit (LCK 014) or similar
- Hach Lange LCK 138 Laton Total Nitrogen (or similar) or discrete/element analyzer
- Discrete analyzer or ion chromatography for ammonium (NH_4^+) measurement (or kit)
- Materials specified in Appendix F (VS/TS measurements)

SAMPLE PREPARATION

When using the kits, the Kaumera sample must be diluted in a way that it fits inside the detection range of the kit. In addition, depending on the pH range of each kit, the pH of Kaumera might need to be increased to be inside the pH detection range. However, make sure that the concentration of cations introduced (e.g., Na) will not cause any further interferences. Make sure that the sample is homogenized before testing.

PROCEDURE

a) Conduct the COD measurement (gO_2/kg) as indicated in the instructions of the kit.
b) Measure the Total Nitrogen via the total N kit or the element analyzer.
c) To obtain organic nitrogen (N_{org}) in g/kg, measure the ammonium concentration and subtract it from the total nitrogen concentration.
d) Perform the ODM (VS) measurement in gVS/kg as described in Section 6.1.
e) Use equations K.1, K.2, and K.3 to determine the stoichiometric coefficients h, o, n (c = 1).

$$h = \frac{308COD + 704N_{org}}{49COD - 64N_{org} + 112ODM} \qquad \text{(K.1)}$$

$$o = \frac{224ODM - 56COD - 304N_{org}}{49COD - 64N_{org} + 112ODM} \qquad \text{(K.2)}$$

$$n = \frac{352N_{org}}{49COD - 64N_{org} + 112ODM} \qquad \text{(K.3)}$$

f) Once the stoichiometric coefficients are known, use equations K.4, K.5, and K.6 to determine the fractions ($mol_x.mol_C^{-1}$) for proteins, lipids, and carbohydrates. Note: assume N_{PR} = 0.26 $mol_n.mol_C^{-1}$ and γ_{LIP} = 5.68 $mol_e.mol_C^{-1}$ (Table 2).

$$\eta_{PR} = \frac{n}{N_{PR}} \qquad \text{(K.4)}$$

$$\eta_{LIP} = \frac{h-2o-3n}{\gamma_{LIP}-4} \quad \text{(K.5)}$$

$$\eta_{CHO} = 1 - \eta_{PR} - \eta_{LIP} \quad \text{(K.6)}$$

SOURCES OF ERROR

- It is important to highlight that these calculations only provide an estimate, as assumptions and generalizations were made.
- The estimated proportion of lipids can be overestimated when there are additional reduced organic compounds present, such as humic substances or alcohols, affecting the carbohydrate proportion as well (Kleerebezem, 2014).
- High concentrations of chloride can interfere with the COD and Total N kits. Check the specific user manual guide for other interferences.

APPENDIX L

Fractionation: Size Exclusion Chromatography (SEC)

This appendix contains the protocol for conducting SEC on Kaumera samples, encompassing detailed instructions for sample preparation, the establishment of a calibration curve for molecular weight determination, and additional considerations that should be taken into account.

MATERIALS

- Minimum of 40 mg dialyzed and freeze-dried EPS
- Buffer solution: 0.15 M NaCl, 50 mM glycine, pH 10 in Milli-Q. For 2 liters, add 17.532 grams NaCl and 7.507 grams glycine, adjust to pH 10 with NaOH, and add Milli-Q water to 2 liters. Note: NaCl is used as high salt concentrations increase solubility. Glycine works as the pH buffer to keep the pH constant throughout testing. Store the solution in a cold room when not in use.
- Washing solution: 0.1M NaOH in Milli-Q water. Dissolve 4 grams in 1 liter of Milli-Q water.
- Storing solution for the column (25% ethanol in Milli-Q water)
- 0.45 µm filter
- Biorad NGC chromatography system
- Cytiva HiLoad 16/600 Superose 6 prep grade column: fractionation range of 5 kDa-5000 kDa
- Cytiva Gel Filtration HMW Calibration Kit

MATERIALS PREPARATION

Rinse all the tubes of the NGC with Milli-Q water.

SAMPLE PREPARATION

a) Conduct dialysis on the Kaumera sample using a 3.5 kDa MWCO membrane against Milli-Q water (Section 5.2.), and subsequently, perform freeze-drying (Section 5.3.).
b) Dissolve 40 mg of EPS in 8 ml of buffer (5 mg/ml) and leave overnight while stirring.
c) Filter the sample through a 0.45 µm filter.

PROCEDURE

a) Connect the 5 ml sample injection loop.
b) Put the tubes A and B in the buffer solution and purge the tubes using a syringe.
c) Set the flowrate to 0.2 ml/min to enable some flow when connecting the column. It is essential that no air enters the column as this can cause damages.
d) To connect the column to the NGC, remove the union connecting the injection valve and UV detector. Detach the cap from the top. Remove the pin from the EtOH stopper from the bottom and push to make the ethanol flow out from the tube from the top. Connect the column to the injection valve making sure there is only liquid-liquid contact. When the flow starts dropping to the bottom, attach the bottom of the column to the UV detector.
e) Dry the column fitting area and continue the slow flow of buffer until no leakage is observed.
f) Conduct an equilibration consisting of 5 column volumes (CV). For example, the Cytiva HiLoad 16/600 Superose 6 prep grade column has a column volume of 120 ml, so 5CV equals 600 ml. The flow rate can be set depending on the time that will elapse until the sample injection the next day (e.g., 16 hours -> 600/960 min = 0.625 ml/min). Equilibration makes sure the column is completely filled with buffer.

g) The next day, warm up the UV detector at 280 nm. However, if needed multiple wavelengths can be selected. Approximately 30 minutes are required for the lamp to warm up.
h) Increase the flow in steps towards 1 ml/min to prevent too much pressure in the system.
i) As the UV warms up, the rack can be filled with tubes to catch the fractionated samples.
j) Once the UV lamp warms, the run can start.
k) Place the filtered sample in a clean syringe.
l) Use the injection needle and remove as many bubbles in the sample as possible.
m) Inject at least 6 ml of sample by slowly putting constant pressure on the syringe. Injecting 6 ml guarantees that the sample injection loop is completely filled and there is no air being added. Leave the syringe on the NGC.
n) Start the run with the software. The elution time equals 1.25 CV, and the process takes 2.5 hours to complete.
o) After the run is finished, collect the fractions and replace the buffer solution with the washing solution and wash it for 1CV for 2 hours at a flowrate of 1 ml/min.
p) After washing, the column can be washed with the storing solution for 1.25 CV (0.9 ml/min for 170 min). Alternatively, washing can be done slowly overnight, similar to the equilibration.
q) To detach the column, turn off the flow, first detach the column from the bottom, press the EtOH stopper slightly to have liquid-liquid contact and close off the end. Detach the top, add the cap on the end and secure the buffer stopper with the spring and lock.
r) Rinse all the tubes of the NGC with Milli-Q water.
s) If required, the EPS fractions can now be dialyzed and freeze-dried.

Figure L.1: Size Exclusion Chromatography (SEC) Setup.

CALIBRATION CURVE

For calibration, the "Gel Filtration HMW Calibration Kit (Cytiva, Product Code 28-4038-42)" can be used. For sets of related compounds, there is a linear relationship between the partition coefficient (K_{av}) and the logarithm of molecular weight. Therefore, to determine the molecular weight range of fractions in an unknown sample, it is possible to construct a calibration curve with standards, in which K_{av} values are plotted against the logarithm of molecular weights. Once the K_{av} values of the unknown sample are calculated, this curve can then be used to determine molecular weights. To ensure the accurate determination of molecular weight, the calibration standards must have the same relationship between molecular weight and molecular size as the substance of interest.

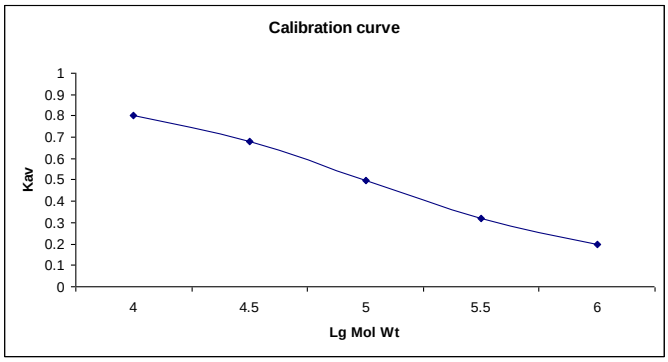


Figure L.2: Example of a calibration curve constructed with protein standards for SEC (Cytiva, 2021).

Some typical protein standards with their respective molecular weights and concentrations used can be found in Table L.1. To obtain such concentrations, the standards are diluted with the same buffer solution as the sample. Peaks are obtained at a wavelength of 280 nm (wavelength commonly targeted to proteins). Since standards consist of only proteins, it is only possible to develop relative protein quantification for Kaumera samples.

Table L.1: Typical protein standards for SEC calibrations. Suggested protein concentrations produce peaks of similar height (Cytiva, 2021).

Protein Standards	Molecular Weight (kDa)	Concentration (mg/ml)
Ovalbumin	44	4
Conalbumin	75	3
Aldolase	158	4
Ferritin	440	0.3
Thyroglobulin	669	5

The molecular weights of the calibration standards are known (Table L.1). Therefore, to create the calibration curve, it is necessary to calculate the K_{av} values for these standards. These calculations are performed using equation L.1. The geometric column volume (V_c) is given by the specifications of the column used (Figure L.3a). From the SEC graph, the elution volume (V_e) for each standard is determined by measuring the volume of the eluent from the point of injection to the center of the elution peak, as depicted in Figure L.3b. To determine the void volume (V_o) (Figure L.3a), a substance such as Blue Dextran 2000 is used. Blue Dextran is a large polymer (MW=2,000 kDa) consisting of dextran molecules covalently linked to a blue dye. Due to its significantly large size, it will not be able access the pores of most columns and thus its elution volume is approximately equal to the column void volume (V_o). Additionally, the blue dye makes it highly visible and easily detectable. By running 5 ml of 0,365 mg/ml of Blue Dextran and determining the elution volume of the first peak in the chromatogram at 254, 280, and 620 nm, it is possible to determine V_o.

$$K_{av} = \frac{V_e - V_o}{(V_c - V_o)} \qquad \text{(L.1)}$$

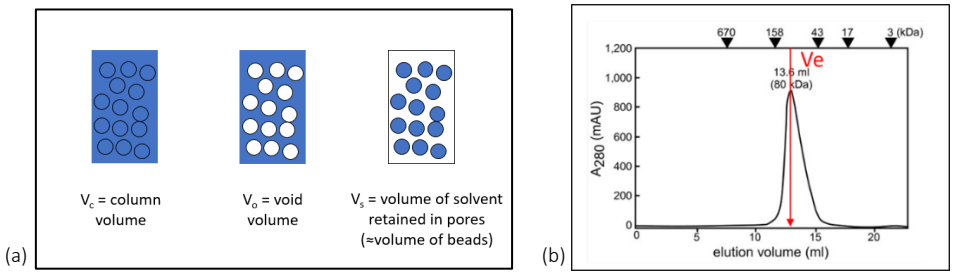


Figure L.3: (a) Graphic representation of V_c, V_o, V_s, (b) V_e.

ADDITIONAL CONSIDERATIONS

The choice of eluent pH has a notable influence on results. As seen in Figure L.4, using a higher pH eluent leads to elevated absorption peaks and a longer elution time. This extended contact between the sample and the column enhances the separation of different fractions. If the suggested pH 10 eluent does not yield satisfactory results, experimenting with a higher pH is a viable option for improving peak separation. pH adjustments can be achieved by adding NaOH.

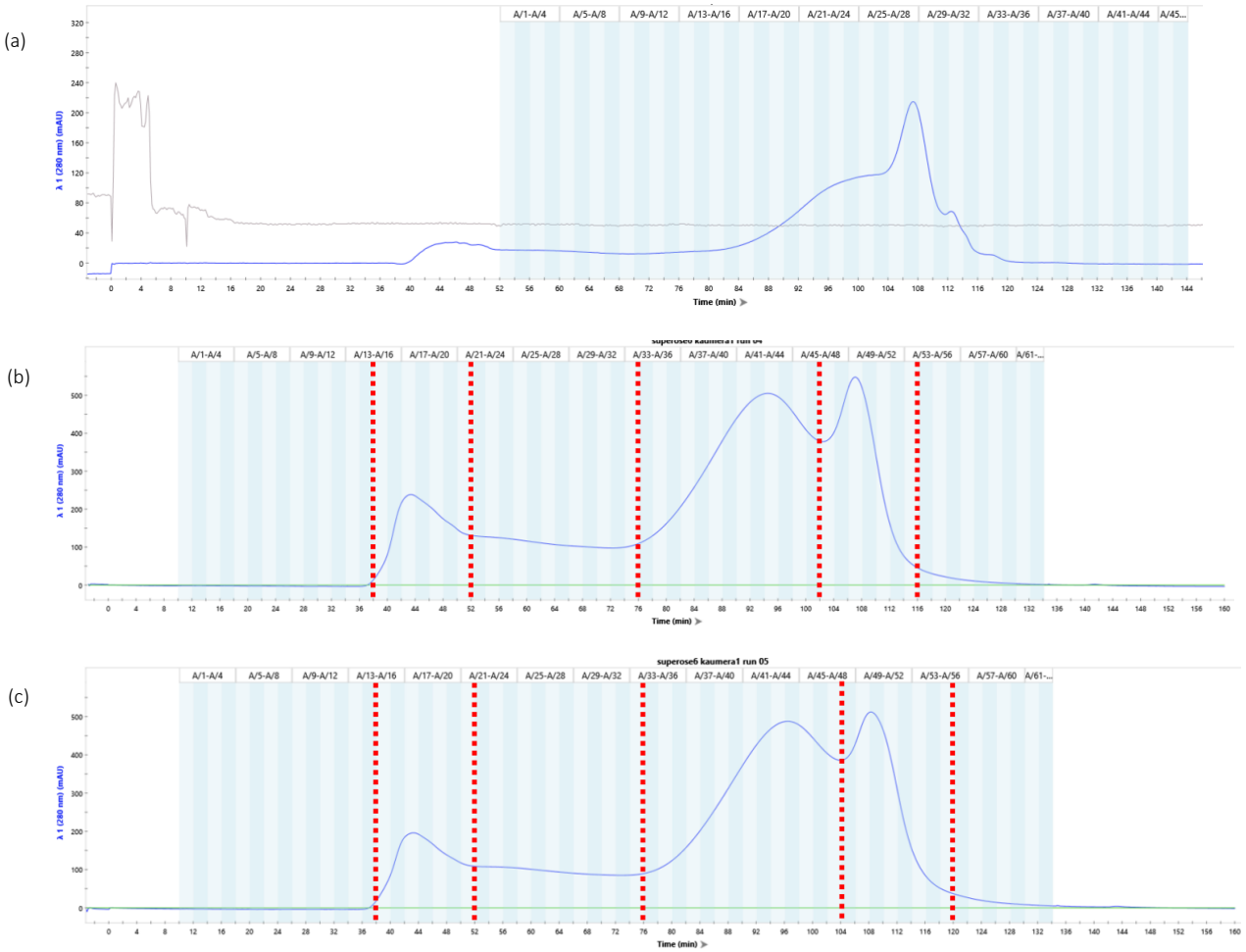


Figure L.4: Absorption spectra for three different runs with different eluents. The dashed lines represent the fraction separation, meaning the different collected samples: (a) eluent 50 mM glycine, 150 mM NaCl at pH 10, (b) eluent 50 mM glycine, 150 mM NaCl at pH 12, and (c) eluent 50 mM glycine, 500 mM NaCl at pH 12.

APPENDIX M

Yield Stress (Rheometer) and Intrinsic Viscosity

This appendix contains the lab method to measure yield stress and viscosity by using a rheometer.

MATERIALS

- 0.6 ml Kaumera sample
- TA DHR-3 rheometer. Depending on the TS% of the sample, the geometry is chosen:
    - If TS% is above 0.3%: a roughened cone of 40 mm diameter is used. The plate is covered with sandpaper to ensure no slip takes place during measurements. The gap distance between the cone and the plate is set to 60-micrometers.
    - If TS% is below 0.3%: a concentric cylinder is used (min volume = 22.5 ml). The cup is filled with the sample and then the bob is introduced. The peak stress is set to 0.1 Pa. Time duration is 10 minutes until a steady (plateau) value for viscosity is reached. In this case, there is no yield stress.

MATERIALS PREPARATION

Before use, the geometry and the pieces are cleaned with water and soap.

PROCEDURE

a) Place the 0.6 ml Kaumera sample in the sandpaper mounted on the plate.
b) Conduct a conditioning ramp. The initial stress is set to 1-5 Pa and the final stress to 0 Pa. The stress decreases linearly for 60 seconds, and 200-400 data points are collected.
c) Afterwards, three stress ramps with different waiting periods (10, 100, and 1000 seconds) are conducted. Like the previous step, stress decreases linearly from 1-5 Pa to 0 Pa in 60 seconds.

APPENDIX N

Acid-Base and Conductometric Titration

This appendix contains the sample preparation, experimental setup, and procedure to conduct an acid-base and conductometric titration for Kaumera samples.

SAMPLE PREPARATION

The idea is to make two titrations, one around 0.005-0.01 M and another at 0.1 M or higher. In general, a titration with low polymer concentration will give a better resolution for the conductivity measurement, while the high polymer concentration will give a better resolution for the pH acid-base curves.

The first step is to dialyze the samples against Milli-Q water with a 3.5 kDa MWCO to reduce the salt content, as described in Section 5.2 (washing as described in Section 5.1 is also possible). With the known concentration of total solids (TS) in Kaumera and using a sodium alginate monomer as reference (=217 g/mol; guluronic or mannuronic acid with sodium), it is possible to estimate how much acidic gel (g) is needed to obtain the desired concentrations. For example, to get a concentration of 0.01 M in a volume of 0.1 L (enough for two 50 ml titrations), 0.217 grams of dry sample is needed (0.01 M * 0.1 L = 0.001 mol; 0.001 mol*217 g/mol = 0.217 grams dry sample). If TS is 5%, 0.217 grams is divided by 0.05, resulting in 4.34 grams of wet sample. The sample is diluted in Milli-Q water, and to this, 1.491 g KCl (=0.2M) are added to screen the charges and solubilize small molecules that could bind to the big polymers otherwise. The dialyzed sample is then acidified (HCl) to pH 2 and titrated with base (NaOH). The base should always have a higher concentration than the sample that is titrated. The conductivity of the analyte at the beginning of the titration is ideally around 0.6-15 mS/cm. If necessary, an additional electrolyte is added, i.e., NaCl, to reach those values.

MATERIALS

- Dialyzed Kaumera suspension (40-200 ml)
- Base titrant with known concentration
- Beakers
- Automatic burette (e.g., Dosimat)
- Magnetic stirrer
- pH/conductivity/temperature probes
- Nitrogen gas line
- Computer and software (e.g., LabVIEW)

EXPERIMENTAL SETUP

To design the titration experiment for the acidic gel Kaumera, it is necessary to decide certain parameters. These parameters should be optimized according to the specific purpose:

- Titrant selection: determine the choice of base and its concentration. While NaOH is a common choice for a strong base, other strong bases such as KOH are also suitable. The concentration of titrant should be higher than the polymer's concentration.
- Total volume of titrant: how much volume of titrant will be added to make sure titration is complete.
- Dispensing rate: how much volume of titrant (ml) will be added per minute.

Note: a higher titrant concentration and high dispensing rate allows for a faster acid-base reaction. However, a lower titrant concentration and/or low dispensing rate may give more data resolution. In addition, a slower titration can increase the risk of atmospheric CO_2 dissolving into the solution.

PROCEDURE

a) Transfer the analyte to a clean beaker with a larger volume than the sample. Add a clean magnet.
b) Rinse the pH/conductivity/temperature probes with milli-Q water, gently dry, and place them inside the beaker.
c) Add a 1/2" yellow needle to the end of the nitrogen gas line. Place the needle above the solution (note: previously, the needle was placed inside the solution, but the bubbles caused the sample to stick to the top of the beaker wall and not be in solution).
d) Take another beaker for flushing the Dosimat automatic burette line and place it under the microtip. Turn on the computer and the software.
e) To clean the Dosimat line, flush a volume of 10 ml at a rate of 5 ml/min. After this is done, check there is no drop of titrant hanging on the dispenser tip and verify the absence of bubbles in the tube. Note: for switching between titrants, for example from NaOH to HCl, the Dosimat should be flushed 3 times, so a total of 30 ml. To be more certain that the Dosimat is not contaminated with NaOH anymore, it is also possible to flush with 30 ml of Milli-Q water first, and then with 30 ml of HCl.
f) Set the dispensing rate, titrant volume, and other essential parameters.
g) Once instrumental values are constant, place the Dosimat microtip on top of analyte, and start the experiment.
h) Analyze how the pH and/or conductivity change as a function of time.

APPENDIX O

Zeta Potential Using Dynamic Light Scattering

This appendix outlines the protocols for measuring zeta potential for Kaumera samples using both ZetaCompact and ZetaNano instruments.

SAMPLE PREPARATION

To measure zeta potential, either dried (powder) or gel Kaumera is dissolved in an alkaline solution. How the solution is prepared —whether it is with Milli-Q water, tap water, or another option—plays a role in determining the conductivity of the solution and therefore will affect the zeta potential measurement. The use of tap water, in specific situations, may be beneficial for introducing bridging ions. The pH of the resulting solution is also a key factor that influences the zeta potential measurement. Hence, when comparing samples, these parameters should be carefully monitored.

Moreover, It is important to consider the particle concentration in the system. If there is a large number of particles present, the measurement may become less reliable due to particle-particle interactions. To ensure accurate velocity measurements, it is advisable to have a relatively low concentration of particles in the Kaumera sample. To achieve this, the Kaumera sample should be diluted further, using the same solvent that was initially used to dissolve Kaumera.

The following steps can be followed for mixing Kaumera at any particular basic pH irrespective of the nature of Kaumera (Kaumera from various sources can have different functional groups, structure, pH nature and as such it is not feasible to create a rigid protocol for dissolving them).

The steps for Kaumera/EPS in pH 12 can be adapted for any basic pH:

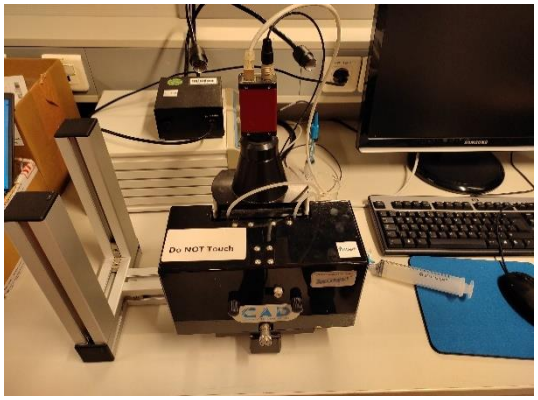
a) Preparing basic pH solutions by mixing NaOH and water (tap).
    a. A small volume of high pH (>13) solution.
        i. Take a small glass beaker with ~100 to 150 ml of water.
        ii. Add 2 or 3 NaOH pellets to it and mix well.
    b. Sufficient amount of pH 12 solution. (We are dissolving the Kaumera/EPS at pH 12)
        i. Take a glass beaker with 500 ml capacity.
        ii. Make 0.01 M NaOH solution (~pH 12) by adding a NaOH pellet to sufficient amount of water.
b) Weighing the amount of Kaumera/EPS (we are making a 500ml solution of 1 g/L)
    a. Take a clean glass beaker with 500 ml measuring capacity.
    b. Tare it to 0 g on a weighing machine.
    c. Add 0.5 g of Kaumera/EPS (equivalent weight) in the beaker.
c) Mixing the solution at pH 12
    a. Add small amounts of pH 12 solution and mix.
    b. Check the pH and add drops of highly basic solution till the pH of the solution reach 12.
    c. Add the remaining pH 12 solution till the total volume becomes 500 ml.

Since Kaumera/EPS is acidic in gel/powdered form, the above mentioned steps can be adapted for pH 7 to 14. In case of dissolving Kaumera/EPS in acidic pH, prepare an highly acidic solution instead of highly basic solution, and proceed as mentioned above.

ZETACOMPACT

This instrument uses a laser to optically track the movement of particles in a dilute suspension in an horizontal capillary cell. The velocity is determined by tracking the motion of the particles when the electric field is applied along the length of the capillary cell.

EXPERIMENTAL SET-UP

(a) 

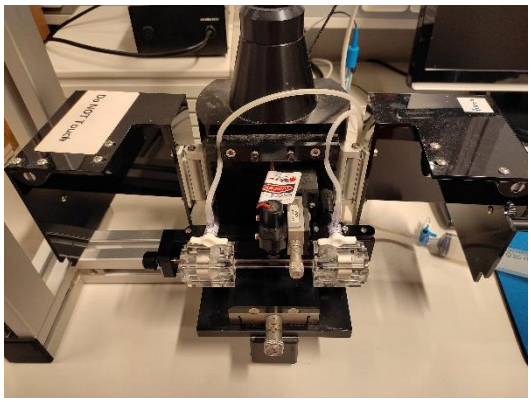
(b) 

Figure O.1: Experimental arrangement for the ZetaCompact system: (a) apparatus featuring a closed compartment. (b) Internal view of the compartment revealing the horizontal capillary cell.

ZetaCompact consists of a closed box with an horizontal capillary cell, a laser source, and a camera. The capillary cell has inlet and outlet pipes that can be closed/opened independently. The cell is filled/emptied by a syringe attached to the outlet pipe.

PROCEDURE

Steps for measuring the zeta potential:

a) Turning on laser and camera
b) Calibration
c) Measurement

Steps for calibration:

a) Open application – Zeta
b) Add particles
c) Set cell wall position
d) Verify cell wall position - Cell profile check
e) Repeat (c) and (d), if necessary, else proceed to measurement.

Adding the particles:

a) Open new calibration file
b) Calibration -> Stationary layer
c) Fill cell with particle suspension
d) Check if sufficient particles are visible on screen
e) Repeat (iii) and (iv) with higher concentration of particles if necessary, else proceed to finding the cell edge.

Setting cell wall position:

a) Open cell cover
b) Move laser position
c) Adjust focus
d) Find a stationary point
e) Set wall position – returns zero position
f) Bring laser to zero position
g) Validate position and close cell
h) Save (calibration is set)

Verify calibration – cell profile check:

a) Measurement -> cell profile -> set profile number = 5
b) Set laser plane to the provided positions (5)
    a. Adjust focus and capture
    b. Repeat for all 5 positions
    c. Start analysis
c) Check if the cell profile is symmetrical, if not repeat from 'setting cell wall position'.
d) Proceed to measurement

Measurement:

a) Set laser plane back to stationary (zero) position
b) Start measurement -> single
c) Export from mobility spectrum

Summary:

a) Clean capillary cell with DI water
b) Add sufficient amount of particles
c) Calibrate/Open calibrated file
d) Move to stationary position and start measurement

ZETANANO

This instrument uses the technique, phase analysis light scattering (PALS), for determining the electrophoretic mobility of the particles in suspension. PALS is based on the classical laser - doppler electrophoresis, but utilizes signal processing of the time domain phase information within the scattered light signal, instead of its frequency spectrum.

EXPERIMENTAL SETUP

a) Open Zetasizer software
    - New folder -> new file
b) Put sample (very diluted) in the U-shaped capillary tube by a syringe
    - The concentrations of the sample should be very low so that the motion of the particles are not influenced by neighbouring particles.

PROCEDURE

Measurement:

1. Measure -> Manual -> Measure Type – Zeta potential
2. Sample (voltage ~ 50V)
    - Temperature – Calibration time – 0 sec
    - Cell – Disposable folded capillary tube (Choose accordingly)
3. Start measurement
4. Select data after closing measurement menu
5. Copy and paste to spreadsheet

Particle Size:

- Measure -> Manual -> Measure Type – Size

APPENDIX P

pH

This appendix provides guidance on cleaning and calibrating pH electrodes for both the extraction process and measurement procedures. Additionally, it offers a detailed, step-by-step guide on measuring the pH of a sample.

MATERIALS

- Sludge/Kaumera sample
- Electrode pH meter (e.g., Digital pH sensor Memosens CPS11E, Endress+Hauser)

Figure P.1: Digital pH sensor Memosens CPS11E (Endress+Hauser)

SAFETY PRECAUTIONS

Wear gloves and work in the fume hood when working with hydrochloric acid, as it is highly corrosive and harmful to eyes and skin.

MATERIALS PREPARATION

- Cleaning the pH electrode:
  a) Rinse the electrode with distilled or deionized water and dry it gently with paper.
  b) Turn on the fume hood and place a jar of 10% w/v HCl in the fume hood.
  c) Place the electrode in the jar containing 10% w/v HCl and wait for 30 minutes. This will dissolve any contamination.
  d) Remove the electrode from the jar and rinse it with water in the fume hood.
  e) Place the electrode in 3 M KCl for at least 15 minutes.
- 2-point pH calibration:
  a) Rinse the electrode with distilled or deionized water.
  b) Take a buffer solution between pH 9 and 10 (depends on the manufacturer) and verify that the electrode indicates the correct pH at the temperature of the buffer solution.
  c) If the pH deviates more than 0.2, calibration is needed.
  d) Place the probe in the pH 7 buffer solution. Ensure the bulb in fully immersed. Once it stabilizes, set the pH meter to the value of the buffer's pH. Repeat the process for the buffer of pH 10.
  e) After calibration, check that the pH electrode in the buffers indicate the correct pH.
     a. If the pH differs from the buffer solution by more than 0.1, repeat the calibration.
     b. If the calibrations fails repeatedly, clean the pH and mentioned above and restart the calibration.
  f) Rinse the pH electrode with water, dry it carefully with paper, and place it in the 3 M KCl until it is needed.
- 3-point pH calibration:
  - If more accurate pH measurements are needed, a 3-point calibration can be done.
  - Follow the instruction above but calibrate with a buffer of pH 7, followed by pH 4, and finalizing with pH 10.

PROCEDURE

a) Submerge a clean and calibrated pH probe into the sludge/Kaumera sample.
b) Allow several minutes for the pH reading to stabilize, then record the value.

c) Remove the pH probe from the sample and rinse it thoroughly with distilled or deionized water to eliminate any residues. Gently dry with paper.
d) Immerse the probe in a storage solution. It is recommended to use a 3 M KCl solution for storage. If 3 M KCl is unavailable, it is also possible to use a pH 4 or pH 7 buffer solution.

ADDITIONAL CONSIDERATIONS

- The pH electrode is an instrument that breaks easily. Handle it with care and avoid contact with the round end of the electrode.
- Always avoid the pH probe from drying out for a long period of time. When not in use, always store it in the 3 M KCl solution.
- The frequency of calibrating a pH meter varies based on its usage and the potential for contamination. Calibration is advisable in the following circumstances: when a new pH electrode is used, after prolonged periods of inactivity, when precise pH measurements are essential, and following pH meter use with highly concentrated solutions.
- For proper usage and storage, read the usage manual of the specific pH probe.

APPENDIX Q

Conductivity

This appendix describes the process of how to measure the conductivity of a sample. This protocol was obtained from: United States Environmental Protection Agency [EPA]. (1982). Method 120.1: Conductance (Specific Conductance, µmhos 25°C) by Conductivity Meter.

MATERIALS

- Self-contained conductivity meter: with an error not exceeding 1% or 1 or 1 µmho/cm, whichever is greater.
- Thermometer: most conductivity meters are already equipped to read temperature.
- Sludge/Kaumera sample

MATERIALS PREPARATION

- *Calibration:* to check the accuracy of the instrument, a standard potassium chloride solution of 0.01 M is used. To prepare the 0.01 M KCl solution, dissolve 745.6 mg anhydrous KCl in Milli-Q water and dilute to 1 liter. At 25°C, the conductivity is of 1413 µmhos/cm. The conductivity at other temperatures are observed in Table Q.1.

Table Q.1: Conductivity of 0.01 M KCl solution at different temperatures (°C) (EPA, 1982).

Conductivity 0.01 M KCl		
Temperature (°C)	µmho/cm	mS/m
21	1305	130.5
22	1332	133.2
23	1359	135.9
24	1386	138.6
25	1413	141.3
26	1441	144.1
27	1468	146.8
28	1496	149.6

PROCEDURE

a) Follow the instructions of the manufacturer for the operation of the instrument.
b) Allow samples to reach room temperature (23 to 27°C) if possible.
c) Measure the conductivity with a sample temperature within 0.5°C. If the temperature of the sample is not 25°C, make temperature correction to convert readings to 25°C as described below.

TEMPERATURE CORRECTIONS

- These temperature corrections are based on the standard KCl solution.
- If the temperature of the sample is below 25°C, add 2% of the reading per degree.
- If the temperature is above 25°C, subtract 2% of the reading per degree.

CONVERSIONS

1 milliSiemens/centimeter [mS/cm] = 100 milliSiemens/meter [mS/m], 1 µmho/cm = 0.1 mS/cm

APPENDIX R

Yield Stress: Viscometer

This appendix describes how to measure the yield stress (on-site) using a Brookfield DV2T Viscometer.

MATERIALS

- Kaumera sample
- Jar for viscosity measurement (Figure R.1)
- Brookfield DV2T viscometer (Figure R.2)
- Spindle V74 from LV spindle set

Figure R.1: Jar for viscosity measurement.

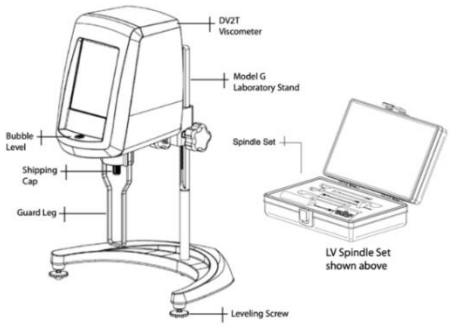


Figure R.2: DV2T viscometer with spindle set.

MATERIALS PREPARATION

a) Make sure the viscometer is levelled with the bubble level (see Figure R.2). If not, adjust by turning the legs.
b) Remove the cap under the viscometer and turn on the DV2T viscometer.
c) Calibrate the device and make sure there are no vibrations.
d) Carefully place spindle V74 on the viscometer as shown in Figure R.3.
e) In the 'main menu' press 'load test' and select the method 'auto temp V74'.

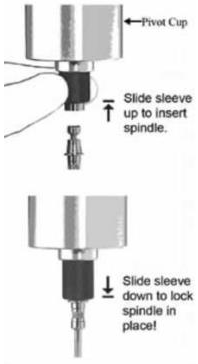


Figure R.3: How to insert the spindle.

Figure R.4: Display screen DV2T.

PROCEDURE

a) Place the jar with Kaumera sample under the viscometer and attach the thermometer with the clip attached.
b) Turn the viscometer down until the line on the spindle reaches the sample. If needed, the jar can be elevated to allow the spindle to reach it.
c) Run the viscometer. The measurement starts automatically when the temperature in the sample has warmed to 20°C. If the room temperature is too cold, it can be heated with the air conditioner. Avoid vibrations during measurement.

d) When it is finished, the highest value measured is the yield stress. Record it (unit = Pa). The results can also be saved as a .CSV file.
e) Remove the spindle and temperature gauge and rinse with water. Dry carefully with paper. Always store the spindle in the spindle set.
f) Place the cap back again on the device and turn it off.

ADDITIONAL CONSIDERATIONS

- When making the measurements, make sure the instrument is on a level surface and there are no vibrations.
- The yield stress is temperature-dependent, which is why it should consistently be assessed at a room temperature of 20°C. Otherwise, record the temperature at which the measurements were taken.
- The yield stress is dependent on the TS content of the sample that is measured. Therefore, when reporting yield always report the TS.