LANDTECHNIK 73(3), 2018, 81–94

DOI:10.15150/lt.2018.3184

LANDTECHNIK
AGRICULTURAL ENGINEERING

# Analysis of carbohydrate degradation during fermentation by an adapted Anthron method

**Manfred Greger, Markus Schlienz, Patricia C. Benito Martin**

To measure the degradation of carbohydrates during fermentation, the photometrical Anthron method has been adapted. The method has been verified in mesophilic batch tests with the model substrates glucose (water-soluble), corn-starch and cellulose (both insoluble in water). First, the content of soluble carbohydrates was measured. For determination of carbo-hydrates in soluble phase, the samples were filtered (syringe PTFE filter with mesh size 0.45 µm) to separate the solution from all types of particles, particulate carbohydrates included. The soluble phase reacted with the Anthron solution for 8 minutes at 100 °C. Afterwards, the coloration reaction was stopped by a quick (5 min) cooling step in ice water to ensure repeatability of the method considering a larger number of samples to be measured. Afterwards samples had been equilibrated at room temperature for at least 30 minutes. Before measurement, the sample had to be diluted to reduce on the one hand the intensity of the background and on the other hand to obtain the measuring range (λ = 625 nm) of soluble carbohydrates of 10 to 100 mg/l.
The total carbohydrate content, which consists of the soluble and particulate fraction, was determined by an additional pre-hydrolysis step (50% sulfuric acid at 100 °C during 8 minutes) before the Anthron reaction was started. To measure insoluble carbohydrates, e.g. starch and cellulose, they had to pass an additional pre-hydrolysis step which is performed before filtration and the subsequent Anthron reaction. This additional step (in the following called “indirect method”) is necessary to measure the part of insoluble respectively polymeric carbohydrates of a sample. The amount of particulate carbohydrates is further calculated by the difference between total and soluble carbohydrates.

**Key words**
Carbohydrate measurement, biogas reactors, Anthron method, pre-hydrolysis

The main components of the substrates applied for anaerobic digestion consist of carbohydrates, proteins and fats (Deublein et al. 2008). Biomass consists mainly of carbohydrates. Carbohydrates themselves can be divided into water-soluble monosaccharides such as glucose and water insoluble polysaccharides such as starch and cellulose. For monitoring of substrate degradation in biogas reactors, it is important to know the degradation rate of the different fractions of carbohydrates. Unpublished research by the authors shows that the method of food analysis according to Weender and van Soest was not appropriate for the observation of the degradation of carbohydrates applied for fermentation processes. In literature, several methods for measuring the carbohydrate content can be found, but

received 21 December 2017 | accepted 19 June 2018| published 25 July 2018

for other forms of applications (Grandy et al. 2000, Loewus 1952, Masuko et al. 2005, Sluiter et al. 2010, Viles et al. 1949, Yan 2014, Yemm et al. 1954).

The adaptation of the photometrical Anthron method to measure the degradation process of carbohydrates seemed to be promising and will be presented hereafter. In this so-called Anthron method, water-soluble saccharides are detected quantitatively with glucose as standard and distilled water as reference. This procedure is applied for the part of soluble monosaccharides (sCH) after an appropriate dilution and without any further intermediate step. If insoluble polysaccharides are present in the sample (particulate carbohydrates), they must be pre-hydrolysed with sulfuric acid of 50% (wt.). This step guarantees that they become soluble and can be measured as dissolved carbohydrates.

This step of carbohydrate de-fractionation corresponds to the total part of carbohydrates (tCH) because potentially present soluble carbohydrates are included in this procedure. The amount of particulate carbohydrates (pCH) can finally be calculated as the difference of total (tCH) minus soluble (sCH) carbohydrates (pCH = tCH − sCH).

## Anthron method, calibration curve with aqueous solution of glucose

Aqueous solutions of glucose (glucose purity > 99.5%, Sigma Aldrich, USA) with different dilutions were mixed with the Anthron solution (0.1 g Anthron, purity > 97%, Sigma Aldrich, USA; dissolved in 100 g sulfuric acid) in the ratio 1 : 2 (0.5 ml sample solution to 1 ml Anthron solution) and heated during 8 minutes in a metallic bloc at 100 °C. Afterwards, the samples were cooled down in ice water to 4 °C. Then, after 30 minutes of equilibration at room temperature, the extinction of the solution was measured at 625 nm with a spectrophotometer (DR 3900, Hach Lange, Germany). A calibration curve was established with glucose stock solution and distilled water as reference (Figure 1). The calibration curve shows the proportionality of glucose concentration and extinction at a wavelength of 625 nm in the range of 10 to 100 mg/l.

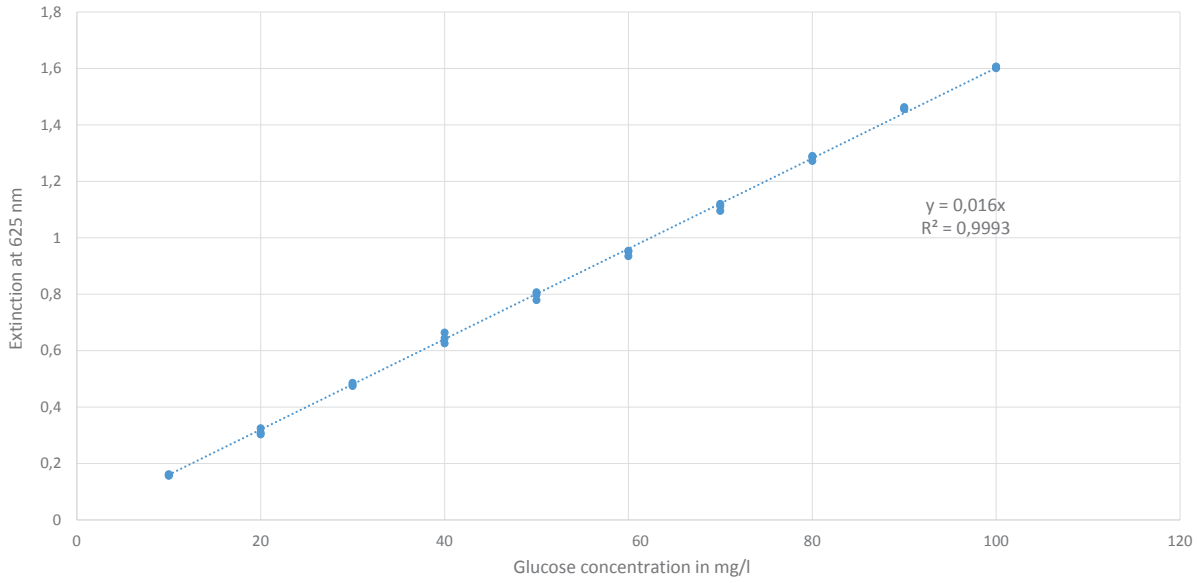


Figure 1: Measurement of the extinction of glucose concentrations in aqueous solution at 625 nm (calibration curve), initial concentration: 0.1 g glucose/l, Anthron solution 0.1% (w/w), reaction time: 8 minutes at 100 °C

## Measurement of the extinction of samples of biogas reactors with and without Anthron reaction for background correction

For the application of the method on digestate samples from bioreactors, a background correction must be applied due to colour intensity of the inoculum. The usual brownish colour of the background is supposed to come from the dissolved parts of lignin and shows a variable extinction at the target wavelength of the Anthron method depending on the factor of sample dilution. Therefore, an appropriate correction of the extinction is necessary to avoid any over-estimation. As a basis of the signal correction the spectrometric value at 625 nm of the initial inoculum is used without the Anthron reaction step and before feeding with the substrate. This value, obtained without hydrolysis reaction, is then compared to the extinction with the Anthron reaction to correlate both values.

Therefore, either the dissolved or the non-filtrated sample of the inoculum can be used depending on the type of substrate (solid/dissolved) and the sample fraction which is to be measured regarding content of carbohydrates. Accordingly, the dissolved phase will be measured with the direct Anthron method and the particulate digestate with the indirect Anthron method (including pre-hydrolysis). For studies of anaerobic fermentation of monosaccharides, the intracellular uptake passes through in a relatively short period. Therefore, the direct Anthron method reflects the uptake kinetics of the active biomass. For fermentation of particulate insoluble carbohydrates, the intermediate step of hydrolysis by exo-enzymes must be introduced to enable the cellular uptake of the hydrolysed intermediate products. Measurements of the concentration of dissolved carbohydrates generally monitor concentrations close to the background, which means that the hydrolysed substances are absorbed without measurable delay and that the process steps involved are located intracellular.

Figure 2 shows the relation between the extinction at 625 nm and the dilution of the dissolved fraction of a sample from a biogas reactor (daily fed with corn silage and a synthetic kitchen waste; semi-batch mode) without (lower curve) and with (upper curve) Anthron reaction step. First, the sample was centrifuged and filtrated with a mesh size of < 0.45 μm. Afterwards, the filtrated phase (including the dissolved substrates and inoculum) was mixed with distilled water in the ratio 1/3 serving as stock solution. This solution was used for further consecutive dilution steps in cuvettes of polystyrene (alternatively quartz glass) to measure spectrophotometrically the background at 625 nm (lower curve in Figure 2).

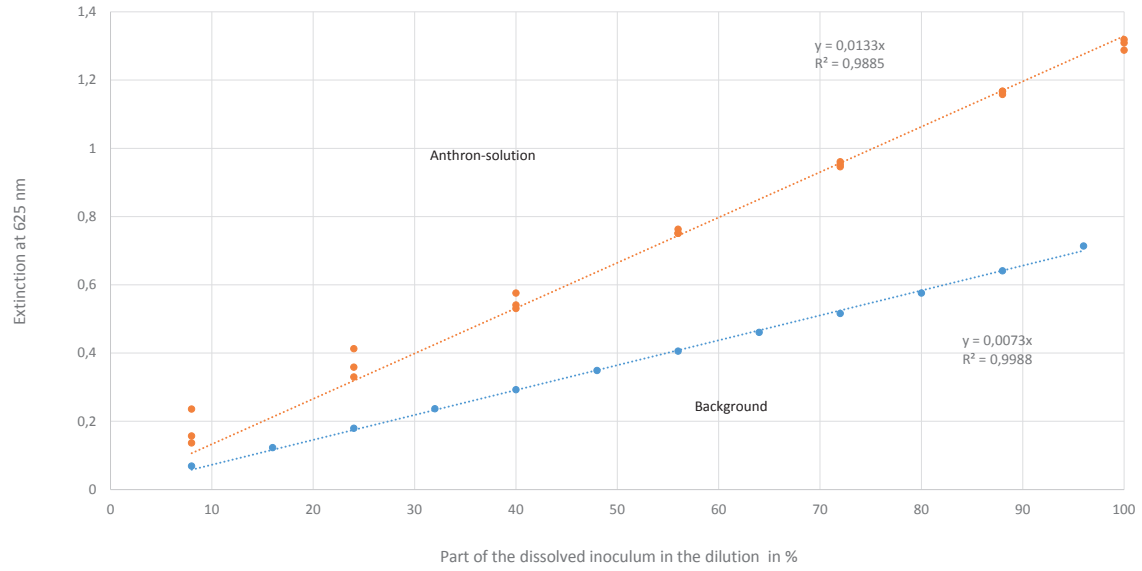


Figure 2: Measurement of the extinction at 625 nm with variation of dilution factor of the filtrate in the range 1:1 (= 100%) to 1:12.5 (8%) without (lower curve, background) and with (upper curve) the Anthron reaction, reaction time: 8 minutes at 100 °C

Figure 3 shows the relation between the extinction of the measured Anthron reaction and the extinction of the background for identical dilutions.

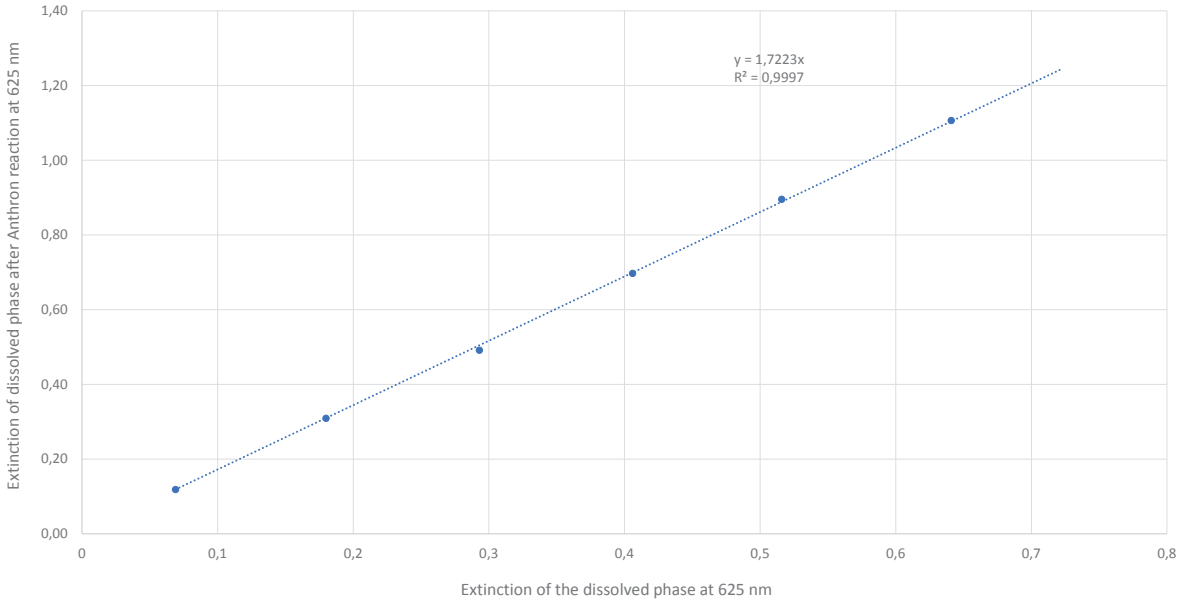


Figure 3: Ratio of extinction of the Anthron reaction (625 nm) and the background signal (625 nm) for identical dilutions of the dissolved phase

Afterwards, the Anthron reaction was performed (reaction time: 8 minutes at 100 °C; upper curve in Figure 2, background corrected). In the range of the highest dilution (< 25%), a systematic deviation from the trend line was observed which could be explained by a change of the pipette used for the dilution.

The slope of the curve in Figure 3 indicates the correction factor for determination of signal background on the extinction value of the Anthron reaction with the prerequisite, that background- and Anthron reaction are measured with the same dilution factor. In case of different dilutions, the values for background correction could be evaluated by interpolation of the graphs in Figure 2.

The following criteria have an influence on the result of the Anthron reaction signal strength and were considered in the method:

- Reaction time with Anthron reagent: the maximum of the extinction is attained after 6 to 8 minutes. The Anthron reaction time was further fixed to 8 minutes.
- Cooling of the mixture of the Anthron solution: the sample of pre-hydrolysis was cooled down in ice water to 4 °C, before it was heated up to 100 °C for the Anthron reaction in the thermo-block. Cooling down of the sample after the Anthron reaction in ice water to 4 °C for 5 minutes and measurement of the extinction after further equilibration to room temperature for 30 minutes (wavelength: 625 nm)
- Stability of the Anthron reactant before spectrometric measurement: in the case of light protected storage, a linear decrease of the signal to 95% of the initial extinction value for a time period of 7 hours was observed. Therefore, the samples have to be measured within one working day.

The validity of the method was further tested with samples of defined concentrations of glucose and inoculum (supplement test). For glucose concentrations in the range 28 to 92 mg/l, the deviation was less than 7% (for immediate measurements). For smaller concentrations as indicated, the deviation raised up to 40%.

## Measurement of particulate carbohydrates of reactor samples with pre-hydrolysis

The direct method cannot be applied for determination of particulate carbohydrates in biogas reactors, fed with solid substrates as corn or grass silages or even microcrystalline cellulose because the method is primary adapted for solid free samples. Therefore, the intermediate step of pre-hydrolysis of the particulate carbohydrates must be introduced before the Anthron reaction step. This procedure is furthermore called indirect method. The particulate carbohydrates are converted into soluble monomers by the pre-hydrolysis step with diluted sulfuric acid at elevated temperature. The pre-hydrolysis leads to an increase of acidity in the following Anthron reaction step and must therefore be calibrated separately in combination with the appropriate substrate.

In a first step, the concentration of sulphuric acid at which the substrate is completely converted into its monomeric composition must be determined considering the appropriate energy input via temperature level and digestion time. Microcrystalline cellulose was used for these experiments because of its purity and its high specific surface which enables a fast hydrolysis. Further, the quick monomerization reflects the influence of the energy input on the coloration and extinction of the Anthron reaction well.

Higher energy input (higher temperature and longer reaction time respectively) in hydrolysis led to increasing brownish discoloration. The process of caramelisation of sugars at higher temperatures

is already known. During measurement of the extinction of the hydrolysates in the wavelength range of visible light, a maximum was detected at 325 nm. Therefore, the extinction of all samples (filtrated < 0.45 µm) were measured at this wavelength to detect a possible influence of the treatment.

Figure 4 shows the influence of the level of sulphuric acid in the pre-hydrolysate in order of digestion time at 100 °C on the intensity of the coloration at 325 nm. The samples were cooled down in ice water immediately and the extinction was measured after 30 min of equilibration at room temperature against distilled water as reference. The variation in intensity of the brownish colour measured at 325nm during hydrolysis shows a clear dependency on the strength of acidity and further on digestion time. Lower acidity level turn out in a retarded augmentation of extinction.

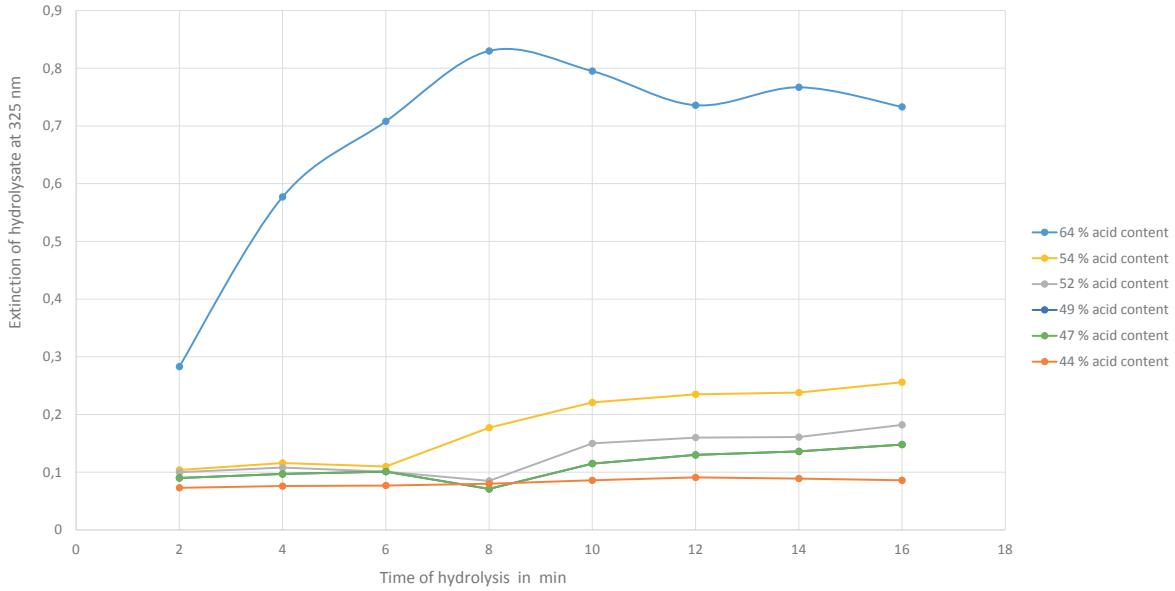


Figure 4: Influence of acidity and digestion time of hydrolysis at 100 °C on the extinction of the pre-hydrolysate.

In the following, the influence of the brownish discoloration on the extinction in the Anthron reaction step will be evaluated. Therefore, the hydrolysates with different sulfuric acid content and different digestion time passed the Anthron reaction in triplicate. In each case (test), filtrated samples ( < 0,45 µm) of 500 µl were mixed with 1 ml of the Anthron solution, cooled down in ice-water to 4 °C and then put to 100 °C during 8 min to perform the Anthron reaction. Afterwards, the samples cooled down again in an ice bath (5 min) After 30 min of equilibration at room temperature, the extinction was measured at 625 nm. Figure 5 shows the extinctions as a function of the total acid content (pre-hydrolysis and Anthron reaction) and the reaction time of the pre-hydrolysis.

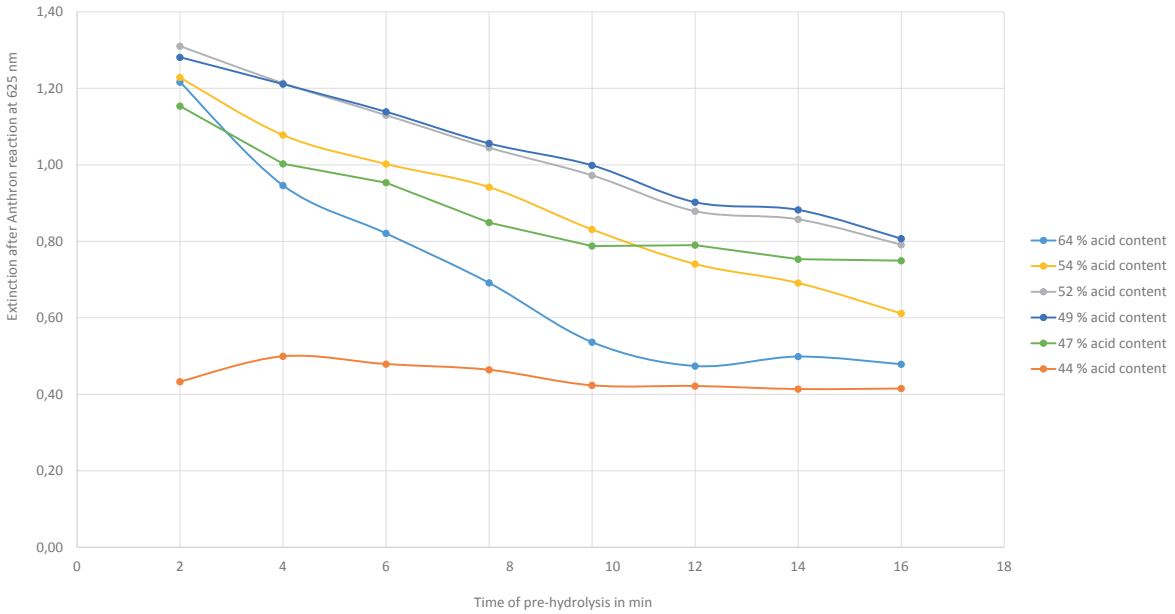


Figure 5: Influence of acid content and reaction time of pre-hydrolysis on the Anthron extinction

The Anthron reaction curves indicate two different phenomena. In the case of low acid content (see the curve for 79,2% total acid content, corresponding to 44% acidity in pre-hydrolysis step), the extinction of the hydrolysate shows nearly a constant value over the whole reaction time. This means that the acid content in pre-hydrolysis step was too low for a complete monomerization of cellulose. The remaining cellulose particles were sieved out in filtration of pre-hydrolysate and were thus not available for the Anthron reaction. The extinction measured therefore was too low. For a total acid content of 80.3% and higher (which is equivalent to 47% acid content in hydrolysate), all extinction values after the Anthron reaction were close to each other for a pre-hydrolysis time of 2 min. With increasing time of hydrolysis and energy input the extinction diminishes depending on the strength of acidity. The brownish discoloration of the hydrolysate correlates reciprocally with the diminishing extinction at Anthron reaction.

The extinction of filtrated pre-hydrolysates (with potential brownish discoloration) can therefore be used as a control parameter to determine the necessary acid content and maximum required energy input. The trend in measured extinction time profiles depends on substrate type and surface characteristics. Microcrystalline cellulose is a model substrate which is fine dispersed and homogeneous compared to other potential substrates types, e.g. silages.

## Monitoring of the degradation of the carbohydrates glucose, starch and cellulose in mesophilic batch-tests

Batch tests at 37 °C with the three model substrates glucose (Glucose purity > 99.5 %, Sigma-Aldrich, USA), corn starch (Sigma-Aldrich, USA) and microcrystalline cellulose (Pharma quality, Euro OTC Pharma GmbH, Germany) were performed. Both concentrations of carbohydrates, either soluble (directly measured) or particulate (indirectly measured via pre-hydrolysis), were measured while anaerobic fermentation. The test was performed in plastic bottles of 0.75-liter net-content. The inoculum had a dry matter (DM) content of 5.4%, an organic dry matter (oDM) content of 3.3%, total inorganic carbon of 15 g/l as $CaCO_3$ and a pH of 8.0. The organic loading rate for all tests was 8 g oDM/l. A detailed description gives Benito (2015).

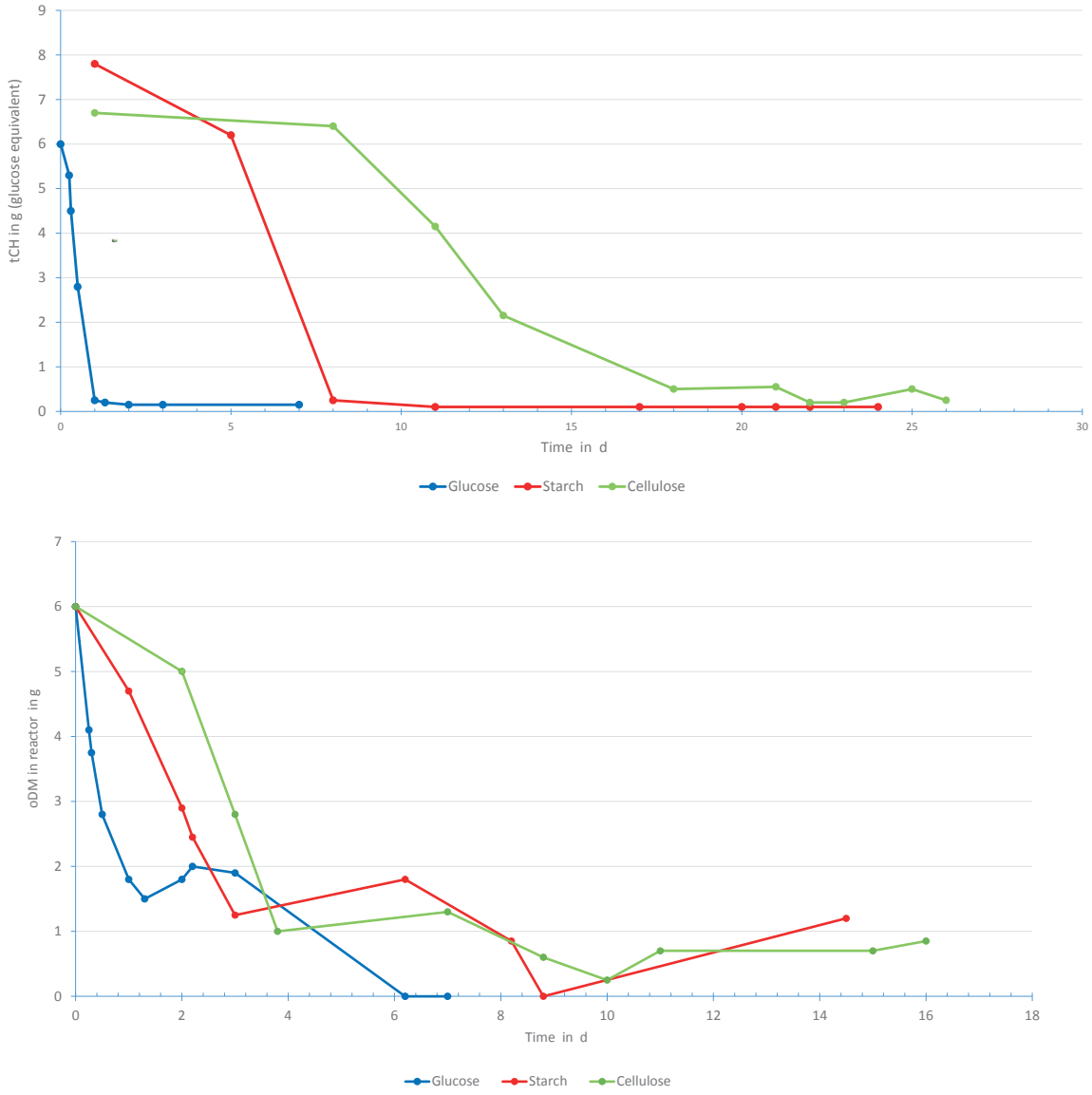


Figure 6: Variation of the total carbohydrates (tCH in g glucose equivalent, upper curve) and the amount of oDM (lower curve) in the fermentation of glucose, cornstarch and cellulose (reactor loading 8 g oDM/l; Benito 2015).

Figure 6 shows the degradation of the total carbohydrate and the experimentally measured content of oDM for the three carbohydrates. The glucose uptake was very quick and completed 1 day after feeding (Figure 6, upper curve). The degradation of cornstarch was delayed by 1 day whereas cellulose degraded mainly after 2 to 4 days and finished after 7 days. In the tests with cornstarch and cellulose, there were not substantial concentrations of dissolved monomers created by hydrolysis. The tests ended after 17 days.

## Measurement of the volatile acids and methane production for balance

For the balance of the tests, it is necessary to measure the production of the volatile organic acids, methane and biogas as well as the oDM content at the end of the test.

Figure 7 shows the production of the intermediate products acetic and propionic acid which were measured with a gas chromatograph (FOCUS GC, Thermo Fisher Scientific, Italy; Benito 2015). Other acids were not detected. In the test with glucose as substrate, a sharp increase of the acetic acid concentration was observed, which reached after approx. 20 h a concentration of 1.44 g/l. The following day, the concentration decreased sharply and finally reached values below the detection limit. The concentration of propionic acid increased with a delay of 6 h, reached the maximum after 44 h and dropped below the detection limit one day after. With starch and cellulose, the increase of acetic and propionic acid concentration was delayed by 1 day. For acetic acid, the maximum values are lower, attained a maximum after 2 days and, fell to values below 50 mg/l 3 days later. Only for propionic acid, all three substrates reached the maxima after 3 days which were smaller for starch and cellulose compared to glucose.

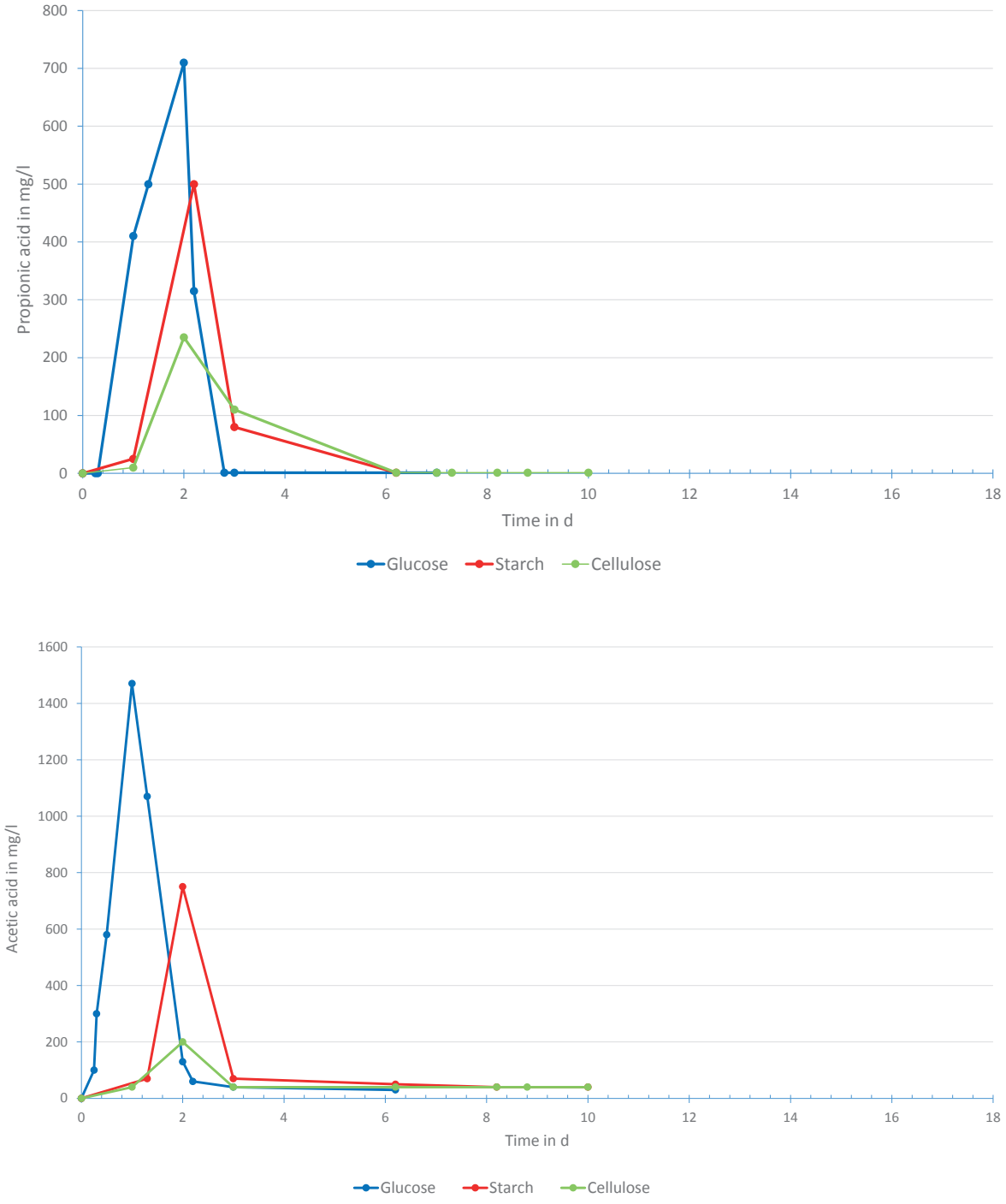


Figure 7: Volatile fatty acids time profile (acetic and propionic acid) in batch tests with glucose, corn starch and cellulose as substrate

The delay of the production of the volatile acids (acetic and propionic acid) for the 3 substrates is also reflected in the variation of pH (Figure 8). In the fermentation trial with glucose, the maximum is reached after 8 h, whereas for starch after 2 days and for cellulose after 3 days.

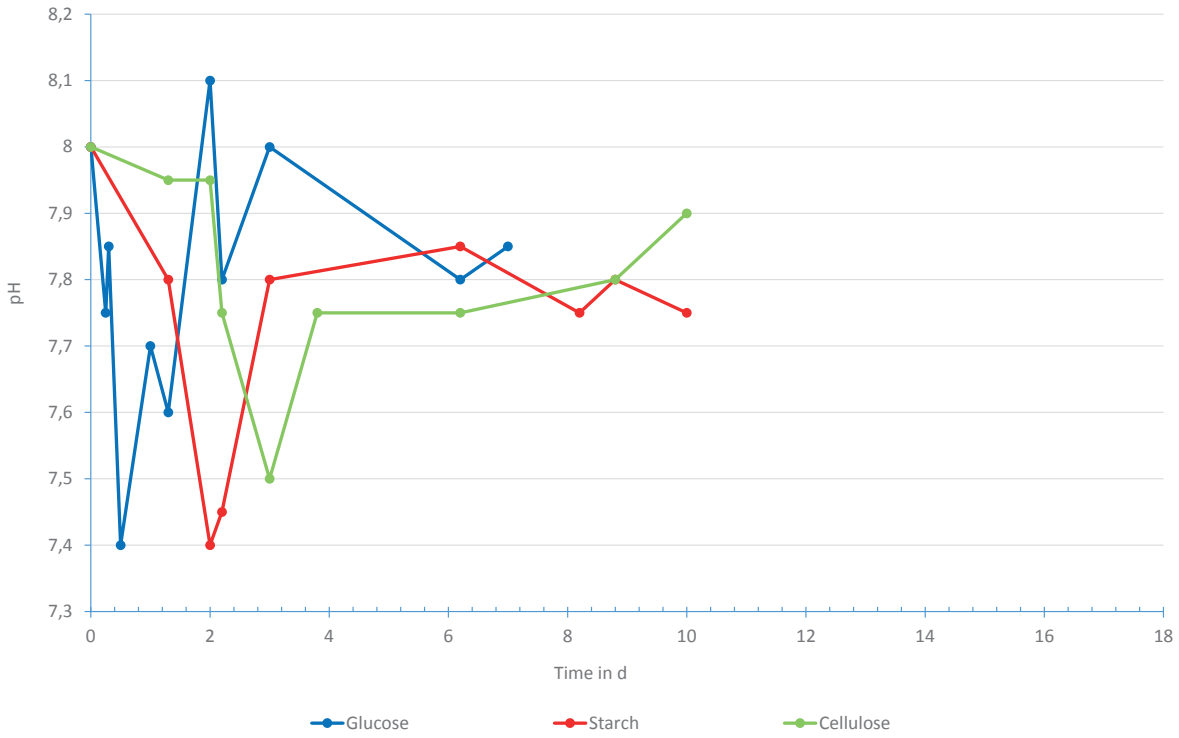


Figure 8: Variation of pH

Figure 9 shows the specific methane production (below) and the methane content in the biogas for the three substrates. Methane and $CO_2$ biogas content were measured with Biogas Monitor BM 2000 (Geotechnical Instruments, GB). Methane production of glucose appeared quite fast and finished after 3 to 4 days. In the case of starch and cellulose, the production of methane started delayed and finished between 6 and 7 days.

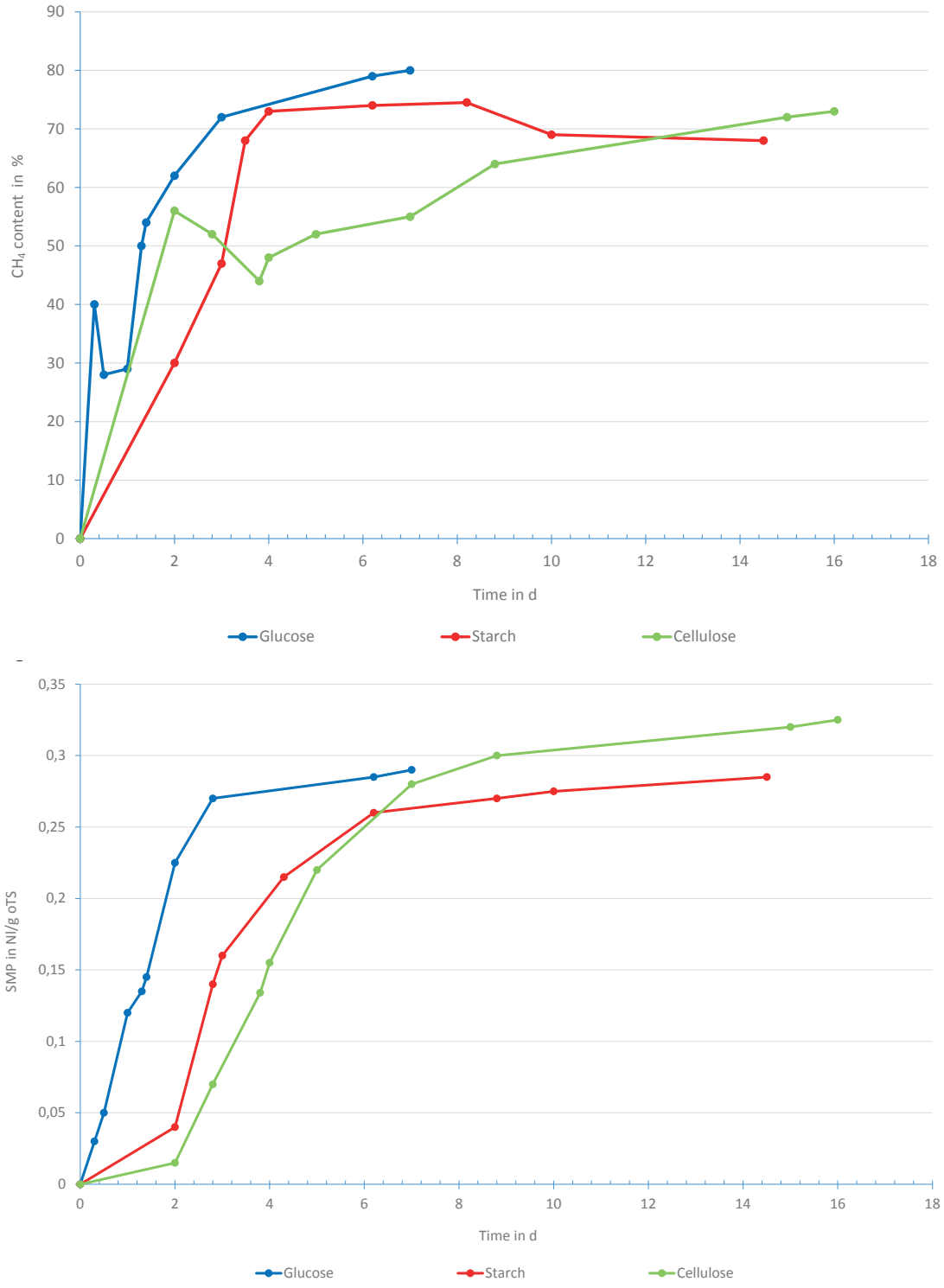


Figure 9: Specific production of methane (SMP, below) and content of methane (up) in biogas for the mesophilic fermentation of glucose, starch and cellulose.

The tests were stopped after reaching the specific methane production of approx. 0.3 Nl/gVS, which was also found as reference in literature (Hansen et al. 2004, Golkowska et al 2013).

The metabolic conversion as a ratio of the experimental value in relation to the theoretical specific potential of methane production (ThSMP) is between 69 and 79% (Table 1). The theoretical value of ThSMP was calculated with Buswell equation (1) (Buswell et al. 1952, Boyle 1976, Raposo et al. 2011, Benito 2015):

$$C_aH_bO_cN_d + \{a - b/4 - c/2 + 3d/4\} \times H_2O \rightarrow$$

$$\{a/2 - b/8 + c/4 + 3d/8\} \times CO_2 + \{a/2 + b/8 - c/4 - 3d/8\} \times CH_4 + d \times NH_3 \quad \text{(Eq. 1)}$$

Table 1: Theoretical specific methane potential and experimentally measured specific methane production for final values or indicated experiment runtime. The ratio of both values is named “recovery”.

| | specific methane potential | | recovery | |
|---|---|---|---|---|
| | ThSMP $Nl(CH_4)/gVS$ | SMP experimental $Nl(CH_4)/gVS$ | % | experimental runtime in d |
| Glucose | 0,373 | 0,285 | 76 | 7 |
| Corn starch | 0,414 | 0,28 | 69 | 14 |
| Cellulose | 0,414 | 0,329 | 79 | 15,8 |

## Conclusions

An adapted Anthron method can be applied to measure the soluble and particular carbohydrates to monitor the degradation of glucose, starch and cellulose in batch biogas reactors.

In further investigations, the method is intended to be applied to continuously run biogas reactors with different substrates. Kitchen waste of different origins is currently of special interest. By separating hydrolysis from color reaction step, it should be verified if the process can be applied to the analysis of digestate under varying background conditions.

## References

Benito Martin, P. C. (2015): Mesophilic Anaerobic Digestion of Lignocellulosic Substrates under different Operating Modes and Extreme Feeding Conditions – Optimisation and Modelling. Dissertation, University of Luxemburg

Boyle, W.C. (1976): Energy Recovery from Sanitary Landfills — A Review. In: Microbiol. Energy Conversion, Eds. Schlegel, H.G.; Barnea, S., Pergamon Press, Oxford, UK, pp. 119–138.

Buswell, A. M.; Mueller, H. F. (1952): Mechanism of Methane Fermentation. Industrial and Engineering Chemistry 44(3), pp. 550-552, cited in Raposo et al. (2011)

Deublein, D.; Steinhauser, A. (2008): Biogas from Waste and Renewable Resources, An Introduction. Weinheim, Wiley-VCH Verlag

Golkowska, K.; Greger, M. (2013): Anaerobic Digestion of Maize and Cellulose under Thermophilic and Mesophilic Conditions – a Comparative Study. Biomass and Bioenergy 56, pp. 545–554

Grandy, A. S.; Erich M. S.; Porter G. A. (2000): Suitability of the Anthrone-Sulfuric Acid for Determining Water Soluble Carbohydrates in Soil Water Extracts. Soil Biology and Biochemistry 32, pp. 725–727

Hansen, T. L.; Schmidt, J. E.; Angelidaki, I.; Marca, E.; Jansen, J. l. C.; Mosbaek, H.; Christensen, T. H. (2003): Method for Determining of Methane Potentials of Solid Organic Waste. Waste Management 24, pp. 393–400

Loewus, F. A. (1952): Improvement in Anthrone Method for Determination of Carbohydrates. Anal. Chemie 24(1), pp. 219–219

Masuko, T.; Minami, A.; Iwasaki, N.; Majima, T.; Nishimura S.-I.; Lee, Y. C. (2005): Carbohydrate Analysis by a Phenol-Sulfuric Acid Method in Microplate Format. Analytical Biochemistry 339, pp. 69–72

Raposo, F.; Fernandez-Cegri, V.; De la Rubia, M. A.; Borja, R.; Béline, F.; Cavinato, C.; Demirer, D.; Fernandez, B.; Fernandez-Polanco, M.; Frigon, J. C.; Ganesh, R.; Kaparaju, P.; Koubova, J.; Méndez, R.; Menin, G.; Peene, A.; Scherer, P.; Torrijos, M.; Uellendahl, H.; Wierinck, I.; de Wilde, V. (2011): Biochemical Methane Potential (BMP) of Solid Organic Substrates: Evaluation of Anaerobic Biodegradability using Data from an International Interlaboratory Study. Journal of Chemical Technology and Biotechnology 86(8), pp. 1088–1098

Sluiter, J. B.; Ruiz, R. O.; Scarlata, C. J.; Sluiter, A. D.; Templeton, D. W. (2010): Composition Analysis of Lignocellulosic Feedstocks. 1. Review and Description of Methods. Journal of Agricultural and Food Chemistry 58, pp. 9043–9053

Viles, F. J.; Silverman, L. (1949): Determination of Starch and Cellulose with Anthrone. Analytical Chemistry 21, pp. 950–253

Yan, X. (2014): High Performance Liquid Chromatography for Carbohydrate Analysis: Chapter 3 in: HPLC Principle, Practice and Procedures, Ed. Zou Y., Nova Science Publisher, pp. 22

Yemm, E. W.; Willis, A. J. (1954): The Estimation of Carbohydrates in Plant Extracts by Anthrone. Biochem. J. 56, pp. 508–514

## Authors

**Markus Schlienz**, **Dr.-Ing. Patricia C. Benito Martin** and **Prof. Dr.-Ing. Manfred Greger** work in the laboratory of Process Engineering in the Faculty of Science, Technology and Communication of the University of Luxemburg, 6, rue Richard Coudenhove-Kalergi, L-1359 Luxemburg-Kirchberg, e-mail: manfred.greger@uni.lu.

## Acknowledgements

The authors thank the University of Luxemburg for the financial support of the project.