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The Influence of Polyphenolic Compounds on Anaerobic Digestion of Pepper Processing Waste during Biogas and Biomethane Production

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Abstract: Pepper processing waste has the potential to be used as a substrate in the process of anaerobic digestion, but because of its high polyphenol content, certain limitations are expected. During the determination of the biodegradability of pepper samples, a biogas potential of 687 L/kg DM was observed, as well as a biomethane potential of 401 L/kg DM. While both the testing of biodegradability and the process in the pilot scale progressed, it was observed that total polyphenol content in both cases decreased. Also, as far as individual polyphenols during the process in the pilot scale are concerned, it can be observed that at the end of the process no procyanidin A2, epicatechin, myricetin, and quercetin were detected. The observed concentration of the ferulic acid on the last day of the process was 0.09 µg/g. Finally, it can be concluded that the presence of polyphenols did not significantly affect the biogas potential of pepper waste. Due to its relatively stable biogas production, as far as biogas production on the pilot scale is concerned, it can be concluded that pepper processing waste has the potential to be used as a substrate for biogas production.

Keywords: *Capsicum annuum*; anaerobic digestion; polyphenols; ferulic acid; procyanidins; epicatechin; myricetin; quercetin

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1. Introduction

Anaerobic digestion of fruit and vegetable waste is a widespread method of waste management because of the high water content that is present in both fruit and vegetables and because of its high biodegradability [1]. Fruits and vegetables are renewable, and if properly converted to methane, they can potentially be used as an energy resource [2]. Also, it is a viable solution for waste management in areas that have abundant agricultural production [3]. Vegetable substrates are reported to achieve higher biomethane potential in comparison to fruit substrates as they are deemed to have more efficient volatile solids removal and are thus more biodegradable in comparison to the fruit ones [3].

Pepper, one of the most popular vegetables around the world, is a member of the *Solanaceae* family, and its taste and flavour are influenced by many factors such as the ripening conditions, genotype, and the colour of the fruit [4]. Pepper is also researched because of its seeds which have promising nutritional properties [5]. It possesses phenolic compounds (secondary plant metabolites) that are highly valued because of their antioxidant activity [6]. Pepper processing generates many byproducts, such as tissues, peels, and seeds, but also processing ingredients such as vinegar can be found [7]. Also, several studies performed research in which the possibilities of exploiting valuable compounds in pepper were examined. For example, it was reported by Razola-Diaz et al. [8] that waste generated during the processing of yellow peppers contains valuable compounds which can be used as ingredients in functional food production.

Polyphenols are organic molecules that are large and complex and are characterised by their large multiple phenol structure units [9]. Polyphenols are known to have numerous biological activities such as antioxidant and antiseptic activities [8]. They are ubiquitous in plants and recently have been a subject of study in foods [10]. Polyphenols such as procyanidins can create complex structures with carbohydrates, fats, and proteins which make the extraction process rather difficult [11].

Anaerobic digestion consists of four main metabolic pathways which are as follows: hydrolysis, acidogenesis, acetogenesis, and methanogenesis, and the main goal of said process is to break down complex substrate to produce biogas [12]. Food waste is an especially convenient substrate for anaerobic digestion due its high biodegradability and high nutrient content [13]. Anaerobic digestion has become an extremely popular method of organic waste management since it produces both methane as an energy source and digestate, which is a liquid fertiliser [14]. However, the usage of pepper in anaerobic digestion is reported to have some limitations, and because of its polyphenol content, a negative influence on anaerobic digestion was observed in several studies [15,16]. Polyphenols can inhibit the fermentation activity of methanogenic microorganisms [9,17]. Hernandez and Edyvean [16] suggested the use of two separate reactors (one for acidogenesis and one for methanogenesis) to reduce the influence of polyphenols on anaerobic digestion. Jiang et al. [18] demonstrated that the addition of yeast extract to the system increased the digester stability and observed that co-digestion with pig slurry is an effective way to maintain the efficiency of anaerobic digestion. The pretreatment of fruit and vegetable processing by-products with various salts and ultrasonic pretreatment can decrease the polyphenol content which in turn increases methane productivity [9]. Also, Chang et al. [19] hypothesised that the addition of red pepper would decrease methanogenesis because of its capsaicin content. However, Fernández-Prior et al. [20] studied the effect of polyphenol extraction on methane production from olive mill waste, where they observed a reduced methane yield by 25% that they attributed to the retention of the biodegradable compounds during the extraction process.

As the data found in the literature suggested, pepper contains polyphenols that are known to be inhibitors of anaerobic digestion, which is why the suitability of using pepper waste in anaerobic digestion is questionable. Since a significantly large amount of pepper waste is produced in the food industry, a pilot scale is deemed to be suitable for studying the influence of polyphenolic compounds on biogas and biomethane production. Furthermore, the total phenolic content will be determined as well as the phenolic profile before and during anaerobic digestion of the said substrate. Obtained results will be compared with the relevant literature to further understand the process and its further applications.

2. Materials and Methods

This research covered two main areas: (1) anaerobic biodegradation of the waste pepper samples obtained during processing in the food industry, and (2) total phenolic content and phenolic profile of the pepper samples, as well as of the digestate obtained during anaerobic degradation.

2.1. Preparation of the Pepper Samples

Pepper samples (*Capsicum annuum*) that were used in this research were the waste obtained during processing in the PODRAVKA Ltd., food industry and it included stems, skins, seeds and pieces of vegetable meat that were left behind after the chopping and cleaning of harvested peppers.

Said samples were sampled on the site of the food industry and transported to the laboratory. Pepper samples were milled in the laboratory mill (Vitamix 300 Pro, Olmstead Falls, OH, USA) to a paste-like state and after this initial analyses of water content, dry matter, volatile matter, and ashes were performed.

Water content and dry matter were performed as follows according to the norm ISO 21660-3:2021 [21]. A dry and clean porcelain evaporating dish was weighed on the

laboratory balance (Mettler Toledo, Greifensee, Switzerland) and the mass was recorded (m_1). After that, ca. 20 g of pepper sample was added to the same porcelain evaporating dish, and the mass was recorded which was the mass of the dish and the sample (m_2). After that, the sample was left to dry in the laboratory drier (Pol-Eko Aparatura SPJ, Wodzisław Śląski, Poland) at 105 °C for 12 h. After that, the dish with the dried sample was left for 20 min to cool in the desiccator after which it was weighed, and the mass was recorded (m_3). To ensure that the drying was complete, a dish with the dried sample was returned to the laboratory drier where it dried for an additional 1 h at 105 °C and was again left for 20 min to cool in the desiccator. The cooled dish with the dried sample was weighed and the mass was recorded (m_4). Since the difference between the masses (m_3 and m_4) of the dried samples was less than 5%, it was concluded that drying was complete. Dry matter (*DM*) and water content (*WC*) were calculated according to the Formulas (1) and (2)

$$\% \, DM = \frac{m_4 - m_1}{m_2 - m_1} * 100 \quad (1)$$

$$\% \, WC = 100 - \%DM \quad (2)$$

The content of volatile matter and ashes were performed as it follows according to the norm EN 15935:2021 [22]. The dried sample in the dish was placed in the laboratory oven (Estherm, Novaki, Croatia) where it was burned for 1 h at 550 °C. After that, the burned sample in the dish was left to cool down and after that, it was weighed, and the mass was recorded (m_5). Volatile matter and ashes were determined according to the Formulas (3) and (4):

$$Ashes \, (\%DM) = \frac{m_5 - m_1}{m_4 - m_1} * 100 \quad (3)$$

$$Volatile \, matter \, (\%DM) = 100 - Ashes(\%DM) \quad (4)$$

In Table 1 the determined properties of pepper samples are shown. In Figure 1, photographs of used samples are shown.

Table 1. Properties of pepper samples.

Pepper Sample	Water Content (%)	Dry Matter (%)	Volatile Matter (% DM)	Ashes (% DM)
Without seeds	91.24 ± 0.342	8.79 ± 0.342	92.61 ± 0.327	7.39 ± 0.327
With seeds	90.16 ± 0.432	9.84 ± 0.432	91.54 ± 0.380	8.46 ± 0.380

(**a**) (**b**)

Figure 1. (**a**) Pepper sample without seeds; (**b**) pepper sample with seeds.

2.2. Biodegradability Determination

The determination of biogas (BP) and biomethane (BMP) potential were performed according to the modified method by Angelidaki et al. [23], since results were expressed

as NL/kg DM. Said experiment was performed to gain insights into how pepper waste is suitable for biogas and biomethane production in the laboratory scale before testing in the pilot scale. Samples for the analysis of total phenols and phenolic profile were taken after third day, as it was estimated that it was a sufficient period for microbial acclimation.

2.3. Continuous Anaerobic Digestion of Pepper Samples in the Pilot Scale

The continuous anaerobic digestion of pepper samples in the pilot scale was performed in the SB reactor (V = 30 L). The temperature in the reactor was kept at 37 °C and the stirring was performed non-stop during the duration of the whole continuous anaerobic digestion. During the first 30 days of the continuous process, the acclimation period of microbial culture occurred. The organic load rate (OLR) is shown in Figure 2. After the acclimation period, the process was tested under different OLR values (see below). The basis of the experiment was as follows. Every day a volume of digestate was removed from the reactor, and an equal volume of pepper paste (the amount depending on the tested OLR value) was added in the reactor to keep the overall volume in the reactor at 30 L. The samples for the analysis of total phenols and phenolic profile were taken after the 30th day, as it was estimated that it was a sufficient period for microbial acclimation. After continuous anaerobic digestion of pepper samples in the pilot scale was performed, the specific production of biogas (SPB) and the specific production of methane (SPM) as well as efficiency was observed. SPB was determined via a mechanically constructed gas meter and SPM was determined via a portable gas detector (Dräger X-am 7000®, DrägerSafety AG & Co. KGaA, Lübeck, Germany). The experiment lasted for 155 days.

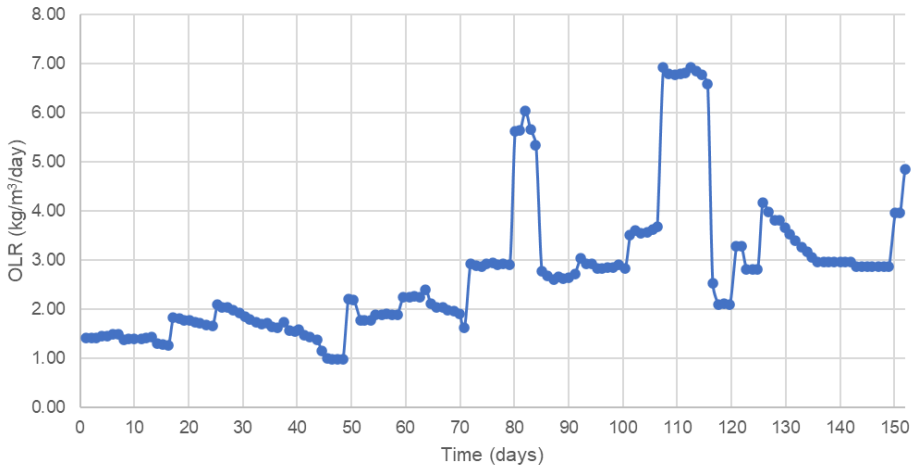

Figure 2. OLR during continuous anaerobic digestion of pepper samples in the pilot scale.

2.4. Extraction of Polyphenols

The samples (pepper waste in the paste-like state and digestate) were frozen rapidly (−80 °C) and after that lyophilised in a lyophiliser (Christ, Alpha LSCplus, Osterode am Harz, Germany). After lyophilisation, the samples were crushed in the laboratory mill on the dimension of ca. 0.5 mm. The obtained powder was extracted with methanol which contained HCl (1%) in the ultrasound bath. After that, centrifugation (10,000 rpm, 10 min) (Thermo Scientific, SL 8R, Suzhou, China) was performed, and the obtained supernatant was filtered through the laboratory filter (0.2 μm). The obtained extract was used in further analysis.

2.5. Total Phenol Analysis

Total phenol content was determined via a spectrophotometer (Shimadzu, UV-1800, Kyoto, Japan). A total of 0.2 mL of the extract was mixed with 1.8 mL of water, and after that 2.5 mL of Folin–Ciocalteu reagent was added, as well as 2 mL of 7.5% Na_2CO_3. After 2 h, absorbance was measured at 765 nm. The results are expressed in mg/L.

2.6. Phenolic Profile Determination

The phenolic profile was determined via HPLC (Shimadzu, pump LC-20AD, column CTO-20AC, photodiode array SPD-M20A, and autosampler SIL-10 Afm, Kyoto, Japan). The mobile phase was a 1% aqueous (purity 98–100%; Scharlau Chemie, Barcelona, Spain) solution of formic acid as solvent A and a methanol (purity $\geq$ 99.9% J.T. Baker, Deventer, The Netherlands) solution of 1% formic acid as solvent B. The injection volume was 10 µL, while the flow of mobile phase was 0.8 mL/min. The temperature of the column and detector was 50 °C. Spectrum recording was performed between 190 and 600 nm, while the detection of components occurred at 280, 320, or 360 nm, depending on the component. The results are expressed in µg/g (of fresh matter). The detection of procyanidin A2 and epicatechin was performed at 280, of ferulic acid at 320, and of myricetin and quercetin at 360 nm. Standards used in phenolic profile determination are listed in Table A1 in the Appendix A.

3. Results

Performed analyses included the determination of total phenolic content and the phenolic profile in pepper samples. Also, changes in the total phenolic content and the phenolic profile during the determination of biogas potential and continuous anaerobic digestion in the pilot scale will be studied. Finally, the content of biogas obtained during anaerobic digestion, as well as the performance parameters of AD will be discussed.

3.1. Total Phenolic Content in Pepper Samples

After the determination of the total phenolic content in pepper samples was performed, the results showed that there was not a significant difference between samples that contained seeds and those that did not. The total phenolic content in the samples that did not contain seeds was 138.62 $\pm$ 5.44 mg/L, while the total phenolic content in the samples that contained seeds was 114.38 $\pm$ 0.54 mg/L. From the comparison of the observed results, pepper without seeds has only ca. 24 mg/L less of total polyphenols which is not significantly different since the continuous process will be conducted on a pilot scale.

3.2. Phenolic Profile in Pepper Samples

After the determination of the phenolic profile in pepper samples was performed, the results showed that ferulic acid (FA), procyanidin A2 (PA2), myricetin (MY), and quercetin (QUE) were identified. From the results shown in Table 2, there is a difference between pepper samples that contain and those that do not contain seeds regarding ferulic acid content. In the pepper samples that did not contain seeds, a concentration of 3.61 µg/g of ferulic acid was detected, while in the pepper sample that did contain seeds, 0.51 µg/g of ferulic acid was detected. The content of procyanidin A2, myricetin, and quercetin in both samples with and without seeds was approximately identical. No epicatechin (EPI) was detected in any of the pepper samples.

Table 2. Phenolic profile in pepper samples.

Pepper Sample	FA (µg/g)	PA2 (µg/g)	EPI (µg/g)	MY (µg/g)	QUE (µg/g)
Without seeds	3.61 $\pm$ 0.02	1.18 $\pm$ 0.01	0.00 $\pm$ 0.00	0.50 $\pm$ 0.02	0.13 $\pm$ 0.04
With seeds	0.51 $\pm$ 0.01	1.21 $\pm$ 0.01	0.00 $\pm$ 0.00	0.68 $\pm$ 0.01	0.14 $\pm$ 0.01

3.3. Determination of Biogas and Biomethane Potential of Pepper Samples

During the determination of the biogas and biomethane potential of pepper samples, the biogas potential of 687 L/kg DM was observed. Also, the biomethane potential of 401 L/kg DM was observed. Said maximal biogas and biomethane potential were achieved on the 10th day of the process (Figure 3).

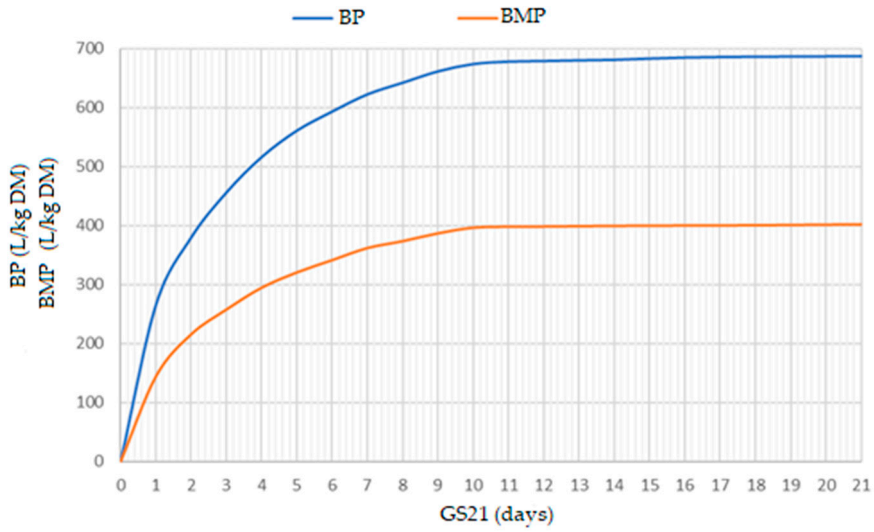

Figure 3. Determination of biogas and biomethane potential of pepper samples.

3.4. Total Phenolic Content during Determination of Biogas and Biomethane Potential of Pepper Samples

After the determination of the total phenolic content in the samples obtained during the determination of biogas and biomethane potential of pepper samples was performed, the results showed that the maximal value of total polyphenol concentration was observed during the third day of the process where the result was 364.77 mg/L. The lowest observed value was on the 20th, and it was 217.46 mg/L. The data from Table 3 show that total polyphenol concentration fluctuates as the process progresses, however, the trend of decreasing total polyphenols is observed.

Table 3. Total phenolic content during determination of biogas and biomethane potential of pepper samples.

Day of the Process	Total Polyphenol Concentration (mg/L)
3	364.77 ± 13.05
5	319.00 ± 0.54
7	307.46 ± 0.54
9	322.46 ± 4.35
12	314.00 ± 0.00
14	294.00 ± 16.32
15	315.92 ± 16.86
16	270.54 ± 0.54
18	239.00 ± 3.81
20	217.46 ± 0.54
21	231.69 ± 3.26

3.5. Phenolic Profile during Determination of Biogas and Biomethane Potential of Pepper Samples

After the determination of the phenolic profile in the samples obtained during the determination of biogas and biomethane potential of pepper samples was performed, the results showed that procyanidin A2 had a maximal concentration on the third day of the process when it was 6.23 μg/g. A minimal observed concentration of procyanidin A2 was observed on the 20th day (1.21 μg/g). Concentrations of ferulic acid, myricetin, and quercetin were lower than the observed concentrations of procyanidin A2 and epicatechin. The maximal observed concentration of epicatechin was 2.03 μg/g (observed on the third day) while the lowest was observed on the twentieth day (0.82 μg/g) (Table 4).

Table 4. Phenolic content during determination of biogas and biomethane potential of pepper samples.

Day of the Process	FA (µg/g)	PA2 (µg/g)	EPI (µg/g)	MY (µg/g)	QUE (µg/g)
3	0.14 ± 0.02	6.23 ± 0.06	2.03 ± 0.13	0.49 ± 0.01	0.30 ± 0.00
5	0.14 ± 0.00	3.70 ± 0.06	1.31 ± 0.15	0.38 ± 0.01	0.23 ± 0.03
7	0.13 ± 0.01	3.63 ± 0.08	1.80 ± 0.04	0.41 ± 0.01	0.24 ± 0.00
9	0.15 ± 0.00	3.68 ± 0.03	1.47 ± 0.20	0.43 ± 0.02	0.22 ± 0.02
12	0.09 ± 0.02	3.84 ± 0.00	1.23 ± 0.00	0.40 ± 0.01	0.28 ± 0.01
14	0.10 ± 0.02	3.22 ± 0.02	1.22 ± 0.19	0.37 ± 0.01	0.22 ± 0.00
15	0.21 ± 0.02	4.63 ± 0.02	1.64 ± 0.13	0.42 ± 0.00	0.26 ± 0.01
16	0.12 ± 0.00	2.60 ± 0.06	1.07 ± 0.05	0.28 ± 0.05	0.23 ± 0.00
18	0.13 ± 0.02	2.19 ± 0.23	1.10 ± 0.33	0.23 ± 0.02	0.21 ± 0.01
20	0.00 ± 0.00	1.21 ± 0.01	0.82 ± 0.08	0.16 ± 0.02	0.14 ± 0.00
21	0.10 ± 0.01	1.59 ± 0.04	1.14 ± 0.11	0.21 ± 0.01	0.16 ± 0.02

3.6. Biogas Production during Continuous Anaerobic Digestion of Pepper Samples in the Pilot Scale

After the continuous anaerobic digestion of pepper samples in the pilot scale was performed, SPB, SPM, as well as efficiency were observed. SPB was between 118 and 934 L/kg DM, while SPM was between 72 and 644 L/kg DM (Figure 4). Efficiency is shown in the graph in Figure 5. Around the 43rd and 145th day, a decline in OLR was performed to test the stability of the system during the circumstances of low organic load. During said period of starvation, SPB and SPM decreased, which is seen from the graph in Figure 4. However, the drop in OLR was not fatal to the system as it fully recovered which is seen from the aftermath increase in efficiency, SPB, and SPM.

The sharp increase in OLR (see in Figure 2) during the 80th–90th day and 105th–110th day was to test the robustness of the system and its ability to recover from the shock. Since there was a significant increase in the OLR during the said periods, consequentially, a rapid increase in efficiency was noted, as well as a rapid increase in SPB and SPM. However, after said shocks, no irreversible decrease in efficiency was noted, and it can be concluded that sharp increases in OLR are not fatal to the system.

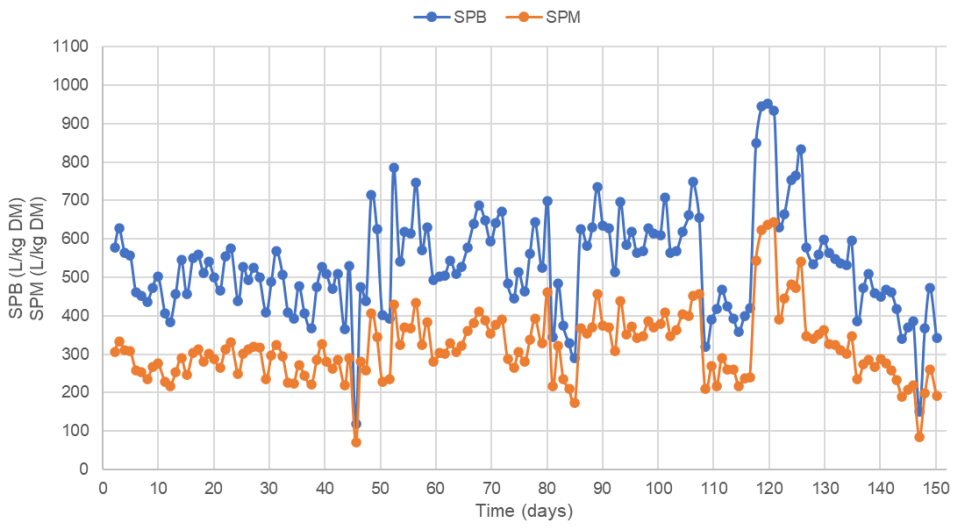

Figure 4. Determination of SPB and SPM of pepper samples during continuous process in the pilot scale.

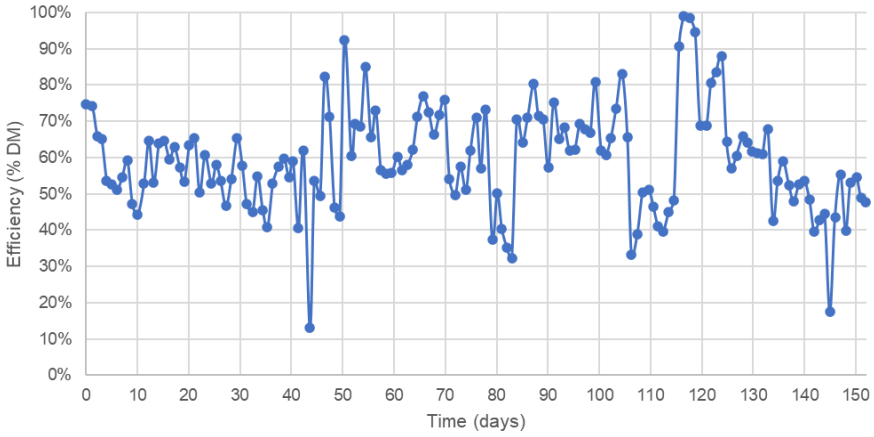

Figure 5. Determination of efficiency of anaerobic digestion of pepper samples during continuous process in the pilot scale.

3.7. Total Phenolic Content during Continuous Anaerobic Digestion of Pepper Samples in the Pilot Scale

After the determination of the total phenolic content in the samples obtained during the continuous anaerobic digestion of pepper was performed, the results showed that the maximal observed value was determined in the 30th of the process and the result was 238.23 mg/L. As the process of anaerobic digestion progressed, total phenolic content decreased; during the whole process a couple of increments in total polyphenol concentration were observed, but neither of them surpassed the maximal. Fluctuations during the whole process can be attributed to the fact that in complex metabolic pathways that occur during anaerobic digestion polyphenols are synthesised, but also degraded which manifests as a drop in concentration.

The lowest observed value was determined on the 147th day of the process and the result was 48.62 mg/L. From the data shown in Table 5, it can be concluded that the decrease in total polyphenols was achieved after the 121st day.

Table 5. Total phenolic content during continuous anaerobic digestion of pepper samples in the pilot scale.

Day of the Process	Total Polyphenol Concentration (mg/L)
30	238.23 ± 10.33
46	144.77 ± 7.61
62	207.46 ± 2.72
72	95.92 ± 1.63
83	98.62 ± 10.88
106	34.00 ± 1.09
118	74.38 ± 12.51
121	92.08 ± 0.54
136	73.62 ± 1.63
147	48.62 ± 6.53
155	54.38 ± 12.51

3.8. Phenolic Profile during Continuous Anaerobic Digestion of Pepper Samples in the Pilot Scale

After the determination of the phenolic profile in the samples obtained during the continuous anaerobic digestion of pepper was performed, the results showed that procyanidin A2 was the most abundant polyphenol observed during the continuous AD of pepper samples. A maximal concentration of procyanidin A2 was observed during the 30th day of the continuous process and the value was 3.68 μg/g, while no procyanidin A was observed during the 155th day.

A maximal concentration of myricetin was detected during the 30th day (0.42 μg/g) while no myricetin was detected after the 83rd day. A maximal concentration of quercetin

was observed during the 72nd day, while no quercetin was detected between 83rd and 118th, as well as after the 136th day.

Ferulic acid was present during the whole continuous process except for the 106th day. The maximal observed concentration of ferulic acid was 0.34 μg/g observed on the 136th day. The highest concentration of epicatechin during the continuous process was observed on the 83rd day when it was 1.5 μg/g. No epicatechin was detected on the 106th, 121st, and on 155th day.

When data from Tables 2 and 6 are compared, myricetin had begun its proper degradation after the acclimation period which occurred after ca. 30 days. As far as procyanidin 2 and epicatechin were concerned, the concentrations fluctuated which can potentially mean that certain metabolic processes occurred in which both procyanidin 2 and epicatechin were synthesised as an intermediate product. Ferulic acid was not detected after the acclimation period, which could indicate that it was used as a substrate in the acclimation period. However, its concentration also fluctuated during the whole process and finally decreased in the last days of the continuous process. The concentration of quercetin increased during the acclimation period and fluctuated when it finally dropped after the 121st day.

Table 6. Phenolic content during continuous anaerobic digestion of pepper samples in the pilot scale.

Day of the Process	FA (μg/g)	PA2 (μg/g)	EPI (μg/g)	MY (μg/g)	QUE (μg/g)
30	0.08 ± 0.02	3.68 ± 0.00	0.84 ± 0.06	0.42 ± 0.02	0.19 ± 0.00
46	0.03 ± 0.01	1.72 ± 0.06	0.45 ± 0.04	0.16 ± 0.01	0.19 ± 0.00
62	0.09 ± 0.00	0.93 ± 0.01	1.10 ± 0.12	0.24 ± 0.03	0.18 ± 0.01
72	0.12 ± 0.02	1.72 ± 0.02	0.46 ± 0.01	0.05 ± 0.01	0.23 ± 0.01
83	0.08 ± 0.01	0.85 ± 0.07	1.50 ± 0.03	0.05 ± 0.01	0.00 ± 0.00
106	0.00 ± 0.00	0.74 ± 0.05	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
118	0.16 ± 0.00	2.12 ± 0.03	0.28 ± 0.02	0.00 ± 0.00	0.00 ± 0.00
121	0.30 ± 0.00	3.56 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.15 ± 0.02
136	0.34 ± 0.01	3.32 ± 0.11	0.25 ± 0.05	0.00 ± 0.00	0.00 ± 0.00
147	0.19 ± 0.00	0.65 ± 0.01	0.27 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
155	0.09 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

4. Discussion

Razola-Diaz et al. [8] performed the ultrasound-assisted extraction after which they analysed the total polyphenol content of both the whole and the edible part of the yellow pepper and observed the concentration of total polyphenols for whole and edible parts in the ranges 3724–3797 μg/g DW and 3241–2767 μg/g DW, respectively. Loizzo et al. [24] analysed different pepper cultivars before and after processing and reported that phenol content in fresh fruit was in the range of 2.3–71.4 mg/g. Also, according to the same research, processing such as boiling leads to a lower content of phenols when compared to fresh fruit. Mohammad Salamatullah et al. [4] reported that the water extract of green, red, and yellow peppers showed polyphenol contents of 30.15, 28.73, and 27.68 mg GAE/g DW, respectively. Hamed et al. [6] observed total phenolic content in various cultivars of peppers during the green and red stages and reported total polyphenol content between 2096 and 5578 μg/g (green stage), as well as between 3760.50 and 7689 μg/g (red stage). When results by Loizzo [24] and Hamed [6], who expressed the total polyphenol content according to fresh matter are compared with ours, it can be seen that our pepper samples contain significantly less polyphenols than those of the aforementioned literature data. The reason for that could be the fact that our substrate was pepper waste, rather than whole peppers. Unfortunately, no relevant literature data were available to our knowledge that contain information about polyphenol profile in digestate during the anaerobic digestion of pepper processing waste which is why the comparison of results could not be conducted.

During the determination of the phenolic profile in the pepper samples, the concentration of quercetin was observed to be ca. 0.13 μg/g. In comparison, Bahorun et al. [25] analysed the quercetin content of various fruits and vegetables and observed the quercetin content in chili peppers of 105 ± 6 μg/g of raw matter. According to the same research, chili

pepper had an observed content of myricetin of 12 ± 2 μg/g of raw matter. In our pepper samples, observed myricetin content was 0.5 and 0.68 μg/g, respectively. No epicatechin was detected in the pepper samples, while Tsanova-Savova et al. [26] analysed the content of epicatechin in various fruits and observed the highest epicatechin content in red grapes (125.6 mg/kg). As far as the ferulic acid content is concerned there is a noted difference between pepper samples that contain and those that do not contain seeds (see Table 2). Manach et al. [27] analysed the polyphenol profile of various fruits and vegetables and reported a concentration of 600–660 mg/kg FM of ferulic acid in eggplant, 300–600 mg/kg FM of quercetin in curly kale, and 10–50 mg/kg FM of myricetin in beans. Procyanidin content was 1.18 μg/g for pepper without seeds and 1.21 μg/g for pepper with seeds. Hammerstone et al. [10] studied procyanidin content in different apple varieties and observed that procyanidin content was between 12.3 and 252.4 mg/serving. From all the presented results that concern the polyphenolic profile in the pepper sample, it can be concluded that relatively low concentrations of ferulic acids, procyanidin A2, myricetin, and quercetin were detected in comparison to other fruit and vegetables. We speculate that the reason for that is the fact that we analysed pepper waste which lacks nutritional value in comparison to the whole pepper. Secondly, another limiting factor could be the extraction process that was performed for the analysis. To maximise the polyphenol yield, further extraction optimisation should be considered.

During the determination of the BP and BMP of pepper samples, a BP of 687 L/kg DM was observed. Furthermore, the BMP of 401 L/kg DM was observed. Research regarding the continuous anaerobic digestion of pepper on the pilot scale is very limited, as is the information about the biogas (biomethane) potential of pepper processing waste. Thus, we focused on obtaining as much information about the biogas (biomethane) potential and anaerobic digestion of vegetables. Ferrer et al. [3] reported BMP 279.8 ± 42.26 mL CH_4 g VS after the anaerobic degradation of pepper in the period of 100 days, and when compared to mixtures where various agricultural waste was tested (tomato, peach, and persimmon), combinations that contained pepper showed the highest final BMP. Furthermore, Nallathambi Gunaseelan [2] studied the BMP of onion skin, cauliflower, potato, and carrots and reported the following findings: the methane yield of rotten tomato was 0.298 m^3/kg VS, of onion skin 0.298 m^3/kg VS, of potato skin 0.267 m^3/kg VS, of cauliflower 0.190–0.331 m^3/kg VS, while the methane yield of carrot was in the range of 0.241–0.309 m^3/kg VS. When the results of the BMP obtained after this research are adapted to the volatile solids, in comparison to other vegetable substrates which are found in the literature, pepper processing waste showed the highest BMP. Also, our BMP results showed higher yield when expressed in volatile solids in comparison to Ferrer et al. [3] The data of BP in vegetable processing waste are scarce and thus cannot be compared to our results.

From the results presented in Table 3, total polyphenol concentration decreases as the process of anaerobic digestion during biodegradability determination progresses, which is also seen if we look at the results of the phenolic profile (Table 4). Also, during the anaerobic digestion in the pilot scale, the decreasing trend of total polyphenols was also noted (Table 5). A significant drop in polyphenol content was visible around the 43rd and 145th day which can be correlated to the lowest observed efficiency which is seen on the graph in Figure 5. Around the 43rd and 145th day, OLR gradually decreased which also confirms the fact that in these periods, SPB and SPM decreased which is seen from the graph in Figure 4.

Also, as far as individual polyphenols during the process in the pilot scale are concerned, it can be seen from the data presented in Table 6 that at the end of the process no procyanidin A2, epicatechin, myricetin, and quercetin were detected. The observed concentration of the ferulic acid on the last day of the process was 0.09 μg/g. Healy and Young [28] studied the anaerobic biodegradation of aromatic compounds to methane and they observed that the degradation of ferulic acid indicated a nearly stoichiometric conversion to CO_2 and CH_4.

It is known that high polyphenol concentrations can lead to lower biogas and biomethane production, but microbial adaptation can increase the tolerance to the inhibitory effect of polyphenols [15]. Battista et al. [9] studied the anaerobic digestion of a non-treated mixture of olive waste and olive wastewater and reported that biogas production was low (0.36 NL/L, 51.6% v/v methane content) which they attributed to the high polyphenol concentration (53.78 GAE/L). As far as individual polyphenols are concerned, Mikucka and Zielinska [17] studied the effect that polyphenols have on anaerobic digestion and reported that ferulic acid had a strong inhibitory effect on the process due to it having an aldehyde group and longer alkyl side chain. Sharma et al. [29] studied quercetin behaviour during the anaerobic digestion of onion waste and reported the drop in quercetin concentration which they attributed to the decomposition of plant tissues. Also, when Wikandari et al. [30] simulated the addition of epicatechin and quercetin in an anaerobic bioreactor they observed that after the addition of 5 g/L of epicatechin and quercetin, methane content was reduced by 90%.

5. Conclusions

During the determination of BP and BMP, the BP of 687 L/kg DM was observed, and the BMP of 401 L/kg DM was observed, which are clear indicators that pepper processing waste has the potential to be used as a substrate in both biogas and biomethane production. After the analyses were performed it can be concluded that the concentration of total polyphenols during the anaerobic digestion of pepper samples had a decreasing trend and their presence did not significantly affect the BP of pepper waste. Contrary to the initial hypothesis, the presence of polyphenols in the pepper processing waste did not have an inhibitory effect on the anaerobic digestion since the continuous process in the pilot scale showed that the efficiency was satisfactory and similar to the conventional anaerobic processes in the existing biogas plants. Also, as far as individual polyphenols during the process in the pilot scale are concerned, it can be established that at the end of the process no procyanidin A2, epicatechin, myricetin, and quercetin were detected; however, only ferulic acid in a relatively low concentration was detected (0.09 μg/g). It can thus be concluded that the certain metabolising of said polyphenols occurred during the experiment. Since the experiment showed that biogas production using pepper waste was quite stable, it can thus be concluded that pepper waste has the potential to be used in biogas production. Also, the use of pepper waste as the source of polyphenolic compounds should be further studied to maximise the full potential of the pepper processing waste.

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Conflicts of Interest: Authors Gregor Drago Zupančič and Anamarija Lončar were employed by the company Croteh Ltd. Author Jasmina Ranilović were employed by the company Podravka Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The companies Hunan Croteh Ltd. and Podravka Ltd. had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Appendix A

See below the standards used during phenolic profile determination; the name of the standard as well as the purity are listed in Table A1, while in Table A2 other used chemicals are listed.

Table A1. Standards used during phenolic profile determination.

Standard	Label	Purity
FA	46278-1g-F	≥99% (HPLC)
PA2	28660-10 mg	≥99% (HPLC)
EPI	E1753-1 g	≥90(%) (HPLC)
MY	70050-25 mg	≥96.0% (HPLC)
QUE	Q4951-10 g	≥95% (HPLC)

Table A2. Other chemicals used.

Chemical	Manufacturer
Methanol	JT Baker, Deventer, The Netherlands (≥99.9%)
HCl	Gram-mol, Zagreb, Croatia
Folin–Ciocalteu reagent	Sigma-Aldrich, St. Louis, MO, USA
Formic acid	Scharlau Chemie, Barcelona, Spain (98–100%)
Na_2CO_3	Gram-mol, Zagreb, Croatia (≥99.5%)

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