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Assessment of different pre-treatment methods for the removal of limonene in citrus waste and their effect on methane potential and methane production rate

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Abstract

The objective of this study was to assess the limonene removal efficiency of three pre-treatment methods when applied to citrus waste and to evaluate their effects on the biochemical methane potential (BMP) and the methane production rate (MPR) using batch anaerobic tests. The methods tested were based on removal (biological pretreatment by fungi) or recovery (steam distillation and ethanol extraction) of limonene. All the treatments decreased the concentration of limonene in the orange peel with average efficiencies of 22%, 44% and 100% for the biological treatment, steam distillation and ethanol extraction, respectively. By-products of limonene biodegradation by fungi exhibited an inhibitory effect also, not making interesting the biological pretreatment. The methane potential and production rate of the treated orange peel increased significantly after applying the recovery strategies, which separated and recovered simultaneously other inhibitory components of the citrus essential oil. Apart the high recovery efficiency of the ethanol extraction process, it presented a favourable energy balance.

Keywords

Citrus waste; anaerobic digestion; limonene; inhibition; fungal pretreatment; ethanol extraction; steam distillation.

1 Introduction

The effect of limonene, the major component of citrus essential oil (CEO), in the batch anaerobic digestion was characterized by Ruiz and Flotats (2016) and it was shown that

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limonene clearly has an inhibitory effect on the process. The half-maximal inhibitory concentration (IC_{50}) value, measured as limonene concentration in the reactor, was found to be 423 mg kg^{-1}, and the minimum inhibitory concentration of limonene was around 200 mg kg^{-1}. Since the usual concentrations of limonene in citrus waste greatly exceed this amount (Ruiz and Flotats, 2014), an inhibitory effect is always expected in the anaerobic digestion of citrus waste. In order to avoid this effect, pretreatments can be applied to remove the limonene from citrus waste. Pretreatment methods reported in the literature adopt two different approaches: removal or recovery (extraction). Pretreatments to remove CEO include aeration and biological treatment (BT). Recovery strategies include centrifugation, steam distillation (SD), steam explosion and liquid extraction with organic solvents.

Aeration and centrifugation have been used for the removal and recovery, respectively, of CEO from citrus peel press liquors, with efficiencies between 78 and 99% (Lane, 1983). However, these treatments are more appropriate for liquids than for solid waste.

BT is mainly based on the activity of fungi. Treatment with fungi enzymes obtained from *Aspergillus* and *Penicillium* was studied by Akao *et al.* (1992). Such treatment favoured the anaerobic digestion, but the authors concluded that the main cause of the CEO removal was not the enzyme pretreatment, but the mixing applied during the process, which lasted for 10 days. Srilatha *et al.* (1995) assessed solid-state fermentation of citrus waste with selected strains of *Sporotrichum*, *Aspergillus*, *Fusarium* and *Penicillium*. This pretreatment reduced the limonene concentration by 55% (on a dry matter basis), which allowed a higher organic loading rate (OLR) to be effective in the subsequent anaerobic digestion process and also produced a higher methane yield than the untreated substrate.

SD is another alternative that has been proven to be effective, reaching a limonene removal yield of 70% in a laboratory set-up, with 1 hour contact time, a water/peel ratio of 6/1

(w/w) and a particle size of <2 mm (Martín *et al.*, 2010). This process is commonly used at the industrial scale for limonene recovery, where yields are usually around 50%.

Steam explosion has also been proposed as a pretreatment to recover limonene prior to the anaerobic digestion of citrus waste. This treatment removed up to 94.3% of the limonene and allowed stable thermophilic anaerobic co-digestion of the treated citrus waste with the organic fraction of municipal solid waste. Digestion of the same mixture was strongly inhibited when the citrus waste was untreated. The investment necessary for steam explosion means that this solution is only affordable for large-scale facilities (Forgács *et al.*, 2011).

Studies of liquid-liquid extraction of limonene from ternary and quaternary mixtures have revealed that ethanol is effective for limonene extraction from an aqueous mixture (Arce *et al.*, 2004; Arce *et al.*, 2005). Solid-liquid extraction using n-hexane has been assessed and shown a good limonene extraction efficiency (80%), but poor methane production in the subsequent anaerobic digestion, due to solvent remaining in the peel (Wikandari *et al.*, 2013).

In addition to the limonene inhibiting digestion, the C/N ratio of citrus waste is often higher than optimum (Ruiz and Flotats, 2014). Lane (1984) pointed out that the co-digestion of citrus waste with animal manures could provide the necessary nutrient balance, thus avoiding the need for supplementation with nutrients.

The most appropriate techniques for pretreating citrus waste in order to avoid the subsequent inhibition of anaerobic digestion by limonene, taking into account the waste characteristics and the limonene removal efficiency reported in the literature, are: solid-state fermentation with fungi, extraction with organic solvents (incorporating solvent removal after the pretreatment) and SD. However, these methods could have other effects on the anaerobic digestion process due to factors such as organic matter removal or temperature effects. The objective of this study was to assess the limonene removal efficiency of these pretreatments

when applied to citrus waste and to evaluate their effects on the biochemical methane potential (BMP). The effect of co-digestion with cow manure (CM) to improve the nutrient balance was also assessed.

2 Materials and methods

2.1 Substrates

Three samples of orange (*Citrus sinensis*) peel (OP) were used. Sample OP1 was prepared from oranges bought in a local market, by peeling the oranges and cutting the peel into pieces of 2-3 cm. Samples OP2 and OP3 were taken on different days from a Spanish juice manufacturing facility where no limonene had been extracted. These latter samples were pieces approximately 3-4 cm long and 1 cm wide. No further preparation was undertaken before the pretreatments. CM for the co-digestion experiments was collected from a Spanish dairy farm.

2.2 Analytical methods

Analysis of total solids (TS), volatile solids (VS), conductivity, alkalinity, phosphorus, potassium, total Kjeldahl nitrogen (TKN), ammonia nitrogen (NH_4^+-N), total and soluble chemical oxygen demand (COD, sCOD) and pH were carried out in triplicate according to the Standard Methods of Analysis (APHA-AWWA-WEF, 2006). Due to the high degree of heterogeneity of the samples, the COD and sCOD results had very large coefficient of variation in all cases and were therefore not considered realistic and useful. Therefore, COD values for the calculation of biodegradability were estimated based on VS content, using the COD/VS ratio of 1.4 obtained from the data of Kaparaju and Rintala (2006).

The individual volatile fatty acids (VFA), acetate, propionate, iso-butyrate, n-butyrate, iso-valerate, n-valerate, iso-caproate, caproate and heptanoate, as well as limonene and α-terpineol, were analysed by gas chromatography (GC) as described in Ruiz and Flotats (2016).

2.3 Pretreatments

Three different pretreatments were applied to the OP: BT, SD and solid-liquid extraction using ethanol (EE). The samples used for the pretreatments were: OP1 and OP2 for BT; and OP3 for SD and EE. All the pretreatment conditions are summarized in Table 1.

Two variations of BT were applied. For one, OP1 was cut and placed in contact with OP naturally infected with fungi of the *Penicillium* genus at room temperature and in contact with the air. The treatment was considered to have finished when the whole sample has been invaded by *Penicillium* (visual control). The other BT applied to OP2 consisted of controlled inoculation of the sample with a mixture of *Penicillium digitatum* and *Penicillium italicum*. This inoculum was prepared by growth in PDA (potato dextrose agar) at 25ºC for 5-7 days. Once the degree of sporulation of the microorganisms was adequate, the spores were purified following ASTM Standard G-21:1996. An Aztek Contempo Airbrush air atomizer (Testors, USA) was used to inoculate OP2, in order to guarantee homogeneous inoculation throughout the whole sample. The PDA was composed of potato infusion (4 g L^{-1}), dextrose (20 g L^{-1}) and bacteriological agar (15 g L^{-1}) and had a pH of 5.6 ± 0.2. The sample was then incubated for one week at 25ºC in partially closed recipients that allowed contact with the air but preventing massive loss of humidity.

SD was applied to OP3 in a laboratory set-up consisting of a round-bottomed flask where the steam was generated, an intermediate vessel where the steam was bubbled through the sample, and a glass refrigerator to condensate the extract. Different contact time, steam flow rate and pressure conditions were applied (see Table 1).

EE was carried out with a mixture of 70% ethanol and 30% water (on a volume basis), with a peel/solvent ratio of 1:10, for 60 minutes. The extraction was performed in a water bath at ambient temperature (EE1) and at 40ºC (EE2). Continuous mixing was applied during the

extraction. After the extraction step, the samples were dried in an experimental horizontal dryer with air at 25ºC and a superficial speed of 1 $m \cdot s^{-1}$ for over 14-15 hours. These conditions were selected to ensure complete removal of residual ethanol and avoid loss of organic matter.

Table 1. Pretreatments applied to the orange peel.

Code	Orange peel sample	Fungal inoculation	Temperature (ºC)	Time	Air and humidity
BT1	OP1	Natural	Ambient	Until complete invasion	Aerobic, no prevention of humidity loss
BT2	OP2	Controlled inoculation with *P. digitatum* and *P. italicum*	25ºC	1 week	Aerobic, with prevention of humidity loss

Code	Orange peel sample	Steam flow rate (mL min^{-1})	Temperature (ºC)	Contact time (min)	Pressure (atm)
SD1	OP3	8	100	60	1
SD2	OP3	8	100	180	1
SD3	OP3	16	100	60	1
SD4	OP3	16	100	180	1
SD5	OP3	8	75.1	120	0.38
SD6	OP3	16	75.1	120	0.38

Code	Orange peel sample	Solvent	Temperature (ºC)	Contact time (min)	Peel/solvent ratio
EE1	OP3	70% ethanol, 30% water	Ambient	60	1:10
EE2	OP3	70% ethanol, 30% water	40	60	1:10

BT: biological treatment; SD: steam distillation; EE: ethanol extraction.

2.4 Biochemical methane potential (BMP) tests

BMP tests were conducted according to the VDI Standard 4630 (VDI, 2006). The experimental set-up and methodology for the analysis of the results were conducted following Ruiz and Flotats (2016). The tests were run in triplicate till constant accumulative methane production, with duration times between 20 and 40 days. Methane production rate (MPR) was calculated as the maximum slope of the cumulative methane production curves. Methane production data are expressed at standard pressure and temperature conditions (0ºC and 1 atm).

Digested material from a full-scale agricultural biogas plant fed with CM and vegetable substrates at the mesophilic temperature range and an organic loading rate of 3 kg_{VS} m^{-3} d^{-1} was used as the inoculum for the BMP tests. When this material was not available, digested material from a pilot-scale digester of 1 m^3 fed with CM at the mesophilic temperature range was used.

In all cases, the initial limonene concentration in the batch anaerobic digesters was below the minimum inhibitory concentration (200 mg kg^{-1}) for batch anaerobic digestion observed by Ruiz and Flotats (2016). Therefore, no inhibition was expected due to the limonene concentration.

Table 2 summarizes all the BMP tests, indicating the substrates and inoculum type, as well as the characteristics and initial concentration used in each test. Due to the inoculum composition, we added neither buffering solution nor nutrients to the digesters.

Statistical analysis was carried out to detect significant differences between the results of the BMP tests. To evaluate whether the average values of two of the tests were different, the t-test (α=0.05) for two samples considering different variances was applied.

2.5 Energy balances

Simplified energy balances were estimated in order to compare the thermal energy required for the pre-treatments and the thermal energy obtained from the methane produced by the anaerobic digestion of the treated OP.

For BT, no comparison was made since it is an ambient temperature treatment and additional thermal energy is not required.

Table 2. Summary of BMP tests.

Substrate	Treatment	Duration (days)	Inoculum				
			Source*	TS (%)	VS (%TS)	ISR**	$gVS_{inoculum}$ L^{-1}
OP1	None	30	PS	6.6	77.8	3.9	24.7
OP1	BT1	30	PS	6.6	77.8	4.0	25.0
M1: OP2 and CM, 1:1 (d.m.)	None	40	FS	4.4	73.1	1.5	14.6
M2: OP2 and CM, 3:1 (d.m.)	None	40	FS	4.4	73.1	1.4	14.3
M3: OP2 and CM, 1:1 (d.m.)	BT2 applied to OP2	40	FS	4.4	73.1	1.6	14.8
M4: OP2 and CM, 3:1 (d.m.)	BT2 applied to OP2	40	FS	4.4	73.1	1.4	14.3
OP3	None	20	FS	6.9	76.8	4.4	26.8
OP3	SD1	20	FS	6.9	76.8	4.7	26.2
OP3	SD2	20	FS	6.9	76.8	5.4	26.2
OP3	SD3	20	FS	6.9	76.8	4.5	26.2
OP3	SD4	20	FS	6.9	76.8	5.6	26.2
OP3	SD5	20	FS	1.6	12.5	0.2	0.9
OP3	SD6	20	FS	1.6	12.5	0.2	1.0
OP3	EE1	20	FS	1.6	12.5	0.2	1.0
OP3	EE2	20	FS	1.6	12.5	0.2	1.0

*Inoculum source: PS: pilot-scale digester; FS: full-scale biogas plant. ISR**: inoculum to substrate ratio, VS basis.

For SD, we considered the consumed energy to be that thermal energy required to increase the water temperature to the boiling point and to evaporate the water,

$$E_{SD} = m_w \cdot (C_{p,w} \cdot \Delta T_w + L_{v,w}), \quad \text{(Eq. 1)}$$

where E_{SD} is the total thermal energy consumed by SD (J); m_w is the mass of water (g); $C_{p,w}$ is the specific heat of water (4.18 J g^{-1} $^oC^{-1}$); ΔT_w is the difference between the ambient temperature and the boiling temperature (^oC); and $L_{v,w}$ is the latent vaporization heat of water (2260 J g^{-1}).

For EE1, the energy is mostly required to evaporate the ethanol at the end of the treatment. So only 10% of the ethanol used for the experiment was considered in the estimations of the energy required, since 90% of the ethanol could be removed by simply letting the sample drain on a filter. For EE2, additional energy consumption is required to heat the ethanol-water mixture to 40^{o}C. The thermal energy necessary for the EE treatments was calculated using the following equation:

$$E_{EE} = m_e \cdot C_{p,e} \cdot \Delta T_{treat} + 0.1 \cdot m_e \cdot (C_{p,e} \cdot \Delta T_{vap} + L_{v,e}), \qquad \text{(Eq. 2)}$$

where E_{EE} is the thermal energy required to remove the residual ethanol after EE (J); m_e is the mass of ethanol (g); $C_{p,e}$ is the specific heat of the ethanol-water mixture used (2.96 J g^{-1} $^oC^{-1}$); ΔT_{treat} is the difference between the ambient and treatment temperatures (^oC), which is 0 for EE1; ΔT_{vap} is the difference between the ambient temperature and the boiling point of the ethanol-water mixture; and $L_{v,e}$ is the latent vaporization heat of the ethanol-water mixture (1267 J g^{-1}).

The thermal energy recovered from the methane produced was calculated for 80% of the maximum methane production obtained in the BMP test, as an estimated achievable value in a continuous process (Ruiz, 2015), taking into account a calorific value of methane of 802.6 kJ mol^{-1} (Perry and Green, 1999) and a thermal efficiency of the boiler of 85%, which is an average of values found in the literature (Jaffrin *et al.*, 2003; Pinto Mariano *et al.*, 2013).

3 Results and discussion

3.1 Biological treatment

The chemical characteristics of OP1, OP2 before and after BT are shown in Table 3.

OP1 underwent treatment BT1, at ambient temperature and with no control of the humidity. Consequently, a loss of water was observed. In case of OP2 (treatment BT2), temperature and humidity control were applied during the treatment, and no loss of humidity was observed.

Although a loss of organic matter was expected due to the consumption of carbohydrates by the fungi used in the pre-treatment (Zheng *et al.*, 2014), no significant variation in the VS concentration was observed after BT.

The increase in the TKN (due to organic nitrogen only), phosphorus and potassium concentrations could be related to the inoculation of the OP samples with *Penicillium* (including the culture medium), or to the humidity or volume variations during treatment.

The limonene concentration of OP2 was 2.19±0.67 g kg^{-1}, showing a removal efficiency after BT2 pretreatment of 22% (on a dry matter basis), which is lower than the 55% obtained by Srilatha *et al*. (1995). This difference could be due to the different microorganisms used.

The results of the BMP test on OP1 before and after applying BT1 are shown in Table 4. The BMP, methane production rate (MPR) and anaerobic biodegradability index (BD) were not statistically different for treated and untreated OP1. The only difference observed was a greater accumulation of hydrogen during the first days of the experiment in the case of untreated OP1 ($2 \cdot 10^{-3}$ atm, partial pressure) at day 5 compared to the treated OP1 ($5.1 \cdot 10^{-4}$ atm) at day 7, but tending to zero in both cases at day 10. BMP tests of untreated and treated OP2 in co-digestion with CM were carried out at different proportions, as indicated in Table

2; the results are presented in Table 4. Four OP and CM mixtures were tested: two in a proportion of 1:1 and two in a proportion of 3:1. One of the mixtures from each pair contained OP that had received treatment BT2. The initial limonene concentration in the batch anaerobic digesters was lower in the mixtures with treated OP, but in all cases it was below the minimum inhibitory concentration (200 mg kg^{-1}) observed in batch anaerobic digestion of cellulose with limonene by Ruiz and Flotats (2016), and no effect was expected due to the limonene concentration.

Table 3. Chemical characteristics of untreated and biologically treated orange peel and cow manure (mean value ± standard deviation).

Parameter (units)	***OP1, untreated***	***OP1 after BT1***	***OP2, untreated***	***OP2 after BT2***	***CM***
TS (g kg^{-1})	183 ± 2	270 ± 4	160 ± 2	110 ± 1	92 ± 1
VS (g kg^{-1} d.m.)	967 ± 6	951 ± 9	960 ± 7	953 ± 8	827 ± 30
EC (µS cm^{-1}, 20ºC)	570 ± 39	n.a.	582 ± 40	588 ± 40	>11700
pH (20ºC)	4.24 ± 0.28	n.a.	4.0 ± 0.3	3.9 ± 0.3	7.6 ± 0.5
N-NH_4^+ (mg kg^{-1})	n.a.	n.a.	269 ± 34	175 ± 22	19.61 ± 2.47
TKN (mg kg^{-1})	1830 ± 183	3780 ± 540	832 ± 96	1947 ± 231	38.6 ± 4.6
Phosphorus (mg kg^{-1})	194 ± 13	424 ± 28	198 ± 139	143 ± 100	6.50 ± 0.45
Potassium (mg kg^{-1})	1171 ± 176	2408 ± 35	1205 ± 18	890 ± 133	21.32 ± 3.20
Alkalinity (mg$CaCO_3$ kg^{-1})	n.a.	n.a.	n.a.	n.a.	842
Acetic acid (mg kg^{-1})	n.a.	n.a.	0	1254 ± 121	22.9 ± 2.3
Propionic acid (mg kg^{-1})	n.a.	n.a.	0	0	6.7 ± 0.6
Iso-butyric acid (mg kg^{-1})	n.a.	n.a.	0	18 ± 1	0.86 ± 0.06
Butyric acid (mg kg^{-1})	n.a.	n.a.	0	0	3.15 ± 0.30
Iso-valeric acid (mg kg^{-1})	n.a.	n.a.	0	14.3 ± 1.1	1.34 ± 0.14
Valeric acid (mg kg^{-1})	n.a.	n.a.	0	0	0.73 ± 0.08

EC: electrical conductivity; n.a.: not analyzed.

The four mixtures were compared in pairs, in order to evaluate the effect of the mixture composition and the effect of BT applied to the OP. No statistically significant difference was found in any of the parameters assessed (BMP, MPR, BD); thus we concluded that BT does not have any beneficial effect on the batch anaerobic digestion of citrus peel under the conditions tested.

3.2 Steam distillation

The results of BMP tests with untreated OP and after the six SD treatments are summarized in Table 4.

All the treated samples except the one with milder conditions (SD1) yielded higher BMP, MPR and BD than the control. At ambient pressure, the best results (36% more BMP and a 76% increase in MPR) were observed for the treatment with the higher steam flow rate and the longest contact time (SD4, see conditions in Table 1). This treatment also removed the most limonene (44%). Partial vacuum conditions, SD5 and SD6, corresponding to low and high steam flow rate respectively, resulted in a 34% and 20% increment in BMP respectively, although no statistically significant for SD5 due to the wide confidence interval obtained, and a 34% and 25% increase in MPR also respectively. The partial vacuum applied in SD5 increased the extraction efficiency compared with the treatment at the same flow rate and higher contact time (SD2). Under these pressure conditions, the higher flow rate applied in SD6 did not significantly increase limonene removal further.

The fact that the maximum efficiency of limonene extraction was achieved for the treatment at the higher steam flow rate and the longest contact time (SD4, see Table 1) is in line with the results of Cannon *et al*. (2013), who observed that longer contact times allow higher

efficiencies in essential oil recovery by SD. The limonene extraction efficiency obtained by Martín *et al*. (2010) was higher (70%), although the results cannot be directly compared due to the different operating conditions of the experiments.

3.3 Extraction with ethanol

The results of BMP tests are summarized in Table 4.

EE led to limonene removal efficiencies of close to 100%. The organic matter concentration (measured as VS) remained constant. After the treatment, the samples were dried at low temperature to evaporate the residual ethanol and then BMP tests were carried out.

Both treatments caused more hydrogen to accumulate (maximum partial pressure values: $9.8 \cdot 10^{-4}$ atm in EE1 at day 3 and $1.6 \cdot 10^{-3}$ atm in EE2 at day 2) than in the untreated sample (maximum value: $1.2 \cdot 10^{-5}$ atm). The treatment at 40ºC showed greater hydrogen accumulation in the biogas at the beginning of the experiment, although with H_2 partial pressure values close to zero at day 10 in all cases.

Both treatments resulted in increments of BMP, MPR and BD. The treatment at 40ºC yielded higher values of MPR and BMP than the extraction at ambient temperature (see Table 4).

3.4 Comparison of treatment results

Given that the effect of the limonene inhibition starts at around 200 mg kg^{-1} (Ruiz and Flotats, 2015) and that the initial limonene concentration in the digesters was below this value, the improvement in the anaerobic digestion yield observed with some of the pretreatments tested here should be attributed to other causes.

BT removed up to 22% of the limonene from the OP. No effect was observed on BMP, MPR or BD.

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Table 4. Summary of treatments' and BMP test results (mean value ± standard deviation). See Table 1 for treatment conditions.

Treatment	VS and limonene concentration in the substrate				Results of the BMP test		
	VS (g kg^{-1} d.m.)	*Limonene (g kg^{-1})*	*Limonene removal efficiency (%)‡*	*Initial limonene concentration in the digester (mg kg^{-1}) ****	*BMP (Nl_{CH4} kg_{VS}^{-1})*	*MPR (Nl_{CH4} kg_{SV}^{-1} d^{-1})*	*Estimated BD (%)*
Biological treatment BT1							
None	967	n.a.	-	n.a.	359 ± 31	40 ± 3	85.7 ± 7.4
BT1	951	n.a.	n.a.	n.a.	374 ± 49 (+4%)	41 ± 6 (+3%)	87.7 ± 11.6 (+2%)
Co-digestion of orange peel and cow manure, with (M3, M4) and without (M1, M2) biological treatment BT2							
M1	894	0.8	-	84.6	335 ± 34	50 ± 5	87.6 ± 8.9
M2	927	1.4	-	122.4	366 ± 34	52 ± 5	91.2 ± 8.4
M3	890	0.5	22****	60.7	343 ± 46	51 ± 6	89.5 ± 12.1
M4	922	0.8	22****	90.6	338 ± 33	41 ± 7	84.0 ± 8.3
Steam distillation							
None	960	2.9	-	112.7	348 ± 1	55 ± 5	83.0 ± 0.2
SD1	962	2.8	0	163.0	325 ± 11 (-7%*)	70 ± 2 (+27%)	77.5 ± 2.7 (-7%*)
SD2	966	1.6	7	90.3	364 ± 73 (+5%)	75 ± 13 (+37%*)	86.8 ± 17.5 (+5%)
SD3	969	1.9	0	110.6	398 ± 59 (+14%)	80 ± 10 (+46%*)	94.9 ± 14.0 (+14%)
SD4	963	0.9	44	52.2	473 ± 24 (+36%*)	97 ± 6 (+76%*)	112.8 ± 5.8 (+36%*)†
SD5	960	1.7	17	95.7	465 ± 83 (+34%)	74 ± 8 (+34%*)	111.0 ± 19.7 (+34%)†
SD6	962	1.9	18	90.5	417 ± 20 (+20%*)	69 ± 3 (+25%*)	99.6 ± 4.8 (+20%*)
Solid-liquid extraction with ethanol							
None	960	2.9	-	112.7	348 ± 1	55 ± 5	83.0 ± 0.2
EE1	954	0.01	99.96	0.0	413 ± 37 (+19%*)	67 ± 3 (+22%*)	98.4 ± 8.9 (+19%*)
EE2	985	0.01	99.82	0.1	465 ± 22 (+34%*)	74 ± 3 (+35%**)	107.9 ± 5.0 (+30%*)†

Values in brackets are increments with respect to the blank (Treatment – None). *Increments with respect to OP3 are statistically significant (α=0.05). ** α=0.1 ***Standard deviations of initial limonene concentration were in the range 0.001-0.003 mg Kg^{-1} and are omitted. The final limonene concentrations in the digesters was negligible (<0.05 mg kg^{-1}) in all cases. ****Removal efficiency of the biological treatment BT2 applied to the orange peel only. †Biodegradability values higher than 100% are attributed to several sources of error such as COD estimation for solid samples, and are explained as complete biodegradation of the substrate. ‡ dry matter basis. n.a.: not analyzed.

The best SD treatment in terms of limonene removal removed as much as 44% of the initial limonene present in the OP, and the associated increments of BMP, MPR and BD were 36%, 76% and 36%, respectively. Other SD treatments, resulting in limonene removal efficiencies similar to those achieved by BT (SD6, with a limonene removal of 18%), showed increases of BMP, MPR and BD (20%-25%), which were not observed for BT.

EE was the best treatment in terms of limonene removal, with efficiencies of nearly 100% in the extractions at both ambient temperature and 40ºC. The improvements in BMP, MPR and BD after EE1 and EE2 were similar to those obtained after SD4, SD5 and SD6.

The increments in BMP and MPR achieved by the most effective treatments in each category (BT, SD, EE) are shown in Figure 1.

The maximum BMP increment observed was around 35%. Similar results were obtained for MPR except in the SD4 treatment, where the MPR increment was higher. This treatment was the most intensive in terms of temperature, contact time and steam flow rate, which could have had an effect on the kinetics of the process, thereby increasing the MPR.

The total organic matter in the OP, measured as VS, remained constant after all the pretreatments. Thus, the improvements in the anaerobic digestion process have to be related either to an increase of the biodegradability of the organic matter in the OP or to the removal of other inhibitory compounds.

The organic matter in OP is highly biodegradable, due to its high sugars content. The fibre content varies between 11% and 42% d.m. (Ruiz and Flotats, 2014). This fraction is less biodegradable, and thermal pretreatments can increase the solubilization of the fibre and increase its biodegradability. However, the temperatures required to achieve this effect are higher than those used in our study (Sambusiti *et al.*, 2013).

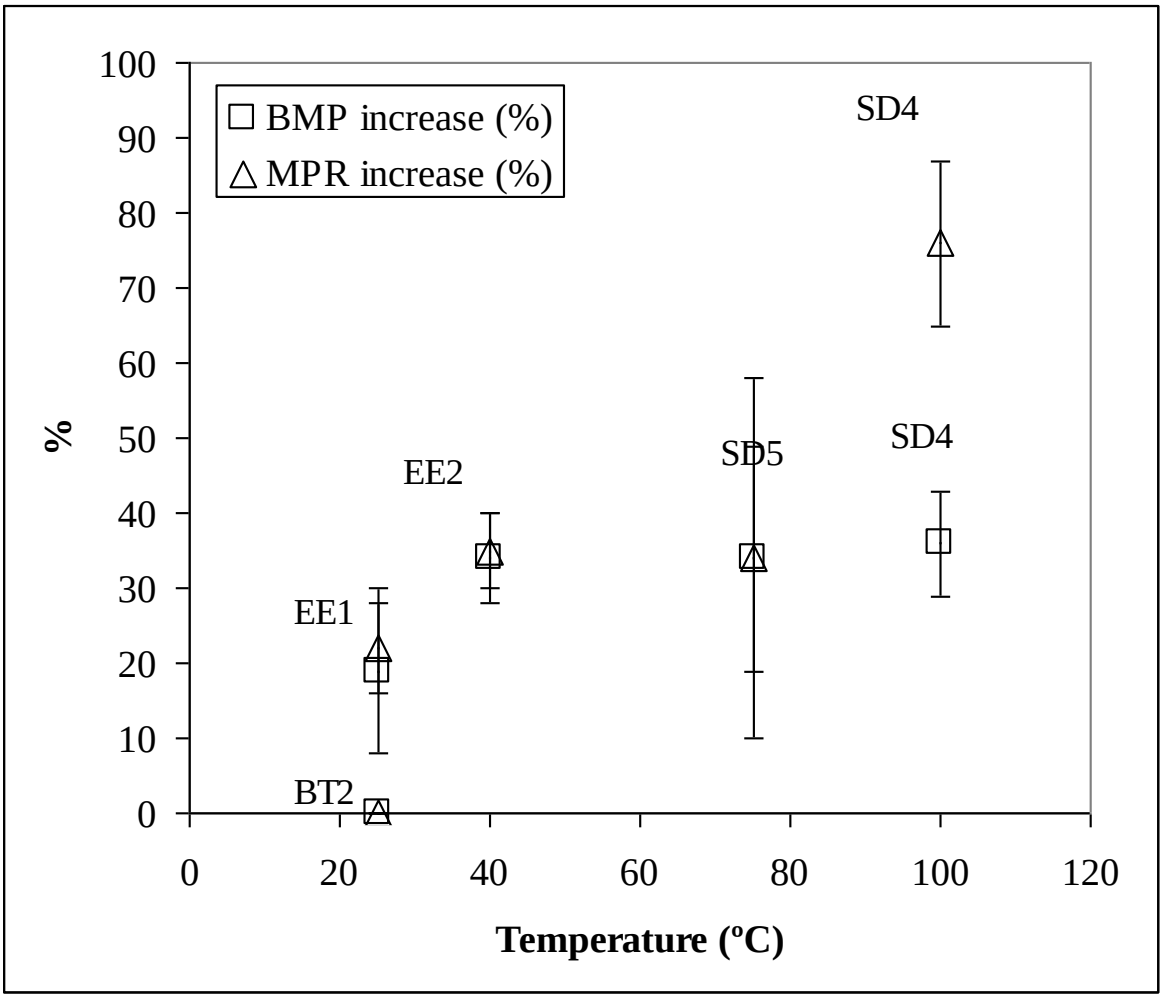

Figure 1. BMP and MPR increase in the batch anaerobic digestion of pretreated orange peel with respect to the untreated samples, depending on the temperature of the pretreatment. Vertical bars represent standard deviation.

Therefore, the remaining possibility is the removal of an inhibitory compound other than limonene. Mizuki *et al.* (1990) observed that the minor compounds present in CEO can have a strong inhibitory effect.

The possible causes could be related to the pretreatment conditions, i.e., the biological process in the case of BT, thermal effects in the case of SD and chemical or thermal effects for EE.

The biodegradation of limonene by *Penicillium digitatum* produces α-terpineol, with bioconversion efficiencies greater than 90% in conditions similar to the pretreatment applied in this work (Badee *et al.*, 2011). Other reported products of the biodegradation of limonene by *Penicillium digitatum* are carveol and carvone (Bowen, 1975). These have been reported to have antimicrobial effects (Ait-Ouazzou *et al.*, 2012; Burt, 2004; Riahi *et al.*, 2013; Viljoen *et al.*, 2005) and therefore could inhibit anaerobic digestion. In particular, the antimicrobial effect of α-terpineol is between 1000 and 5000 times greater than that of limonene, in accordance with their minimum inhibitory concentrations for microorganisms such as *E. coli* and *S. aureus* (Cosentino *et al.*, 1999; Sonboli *et al.*, 2005; Di Pasqua *et al.*, 2006). This biotransformation was observed in our experiments; during BT, the limonene present in the OP was transformed to α-terpineol with 67% efficiency. However, although no increase of the BMP, MPR or BD was observed, no decrease was detected either. A possible increase in the BMP of the co-digestion mixtures with pretreated OP could have been masked by an inhibitory effect of the α-terpineol, which was not completely degraded by the end of the batch anaerobic digestion in the case of mixture M4 (see Figure 2).

SD removed other minor components of the essential oil that have been proven to strongly influence the inhibitory effect (Lane, 1980; Mizuki *et al.*, 1990). This would explain the fact that the BMP, MPR and BD increased as long as the treatment time and steam flow rate increased. Rezzoug and Louka (2009) observed that the CEO obtained by steam distillation (2 h contact time, water/peel ratio 7/1, w/w) contained 94.4% limonene, 1.3% myrcene, 0.5% α-pinene, 0.39% linalool and 0.38% β-pinene (all w/w). Blanco Tirado *et al.* (1995) performed steam distillation with 1-1.5 kg orange fruit peel, with 1 $kg \cdot h^{-1}$ steam at 1.1 atm and obtained 0.17% CEO. Limonene was the main component (91.03%-92.57%). Other compounds were terpinolene (1.83%-2.61%), n-octanal (1.50%-1.64%), β-pinene (0.63%-1.05%), γ-terpinene

(0.41%-1.09%), α-pinene (0.28%-0.32%), comphene (0.27%-0.35%) and decanal (0.11%-0.35%). Under 0.25% of the contents were geraniol, geranial, neral, terpinen-4-ol, nerol, δ-elemene, 3-carene, isopulegol, δ-cadinene, sabinene, α-phellandrene, 1,4-cineole, *trans*-β-ocimene, n-octanol, *cis*-epoxylimonene, perillaldeyde, β-caryophyllene, germacrene D and β-myrcene. (All percentages are GC peak areas.) To the best of our knowledge, the quantification of the inhibitory effect of these minor components, compared to that of limonene on the same microorganisms, has not been reported; but the studies by Lane (1980) and Mizuki *et al*. (1990) demonstrate that the inhibitory effect of CEO (containing the minor components) is higher than the inhibitory effect of limonene alone.

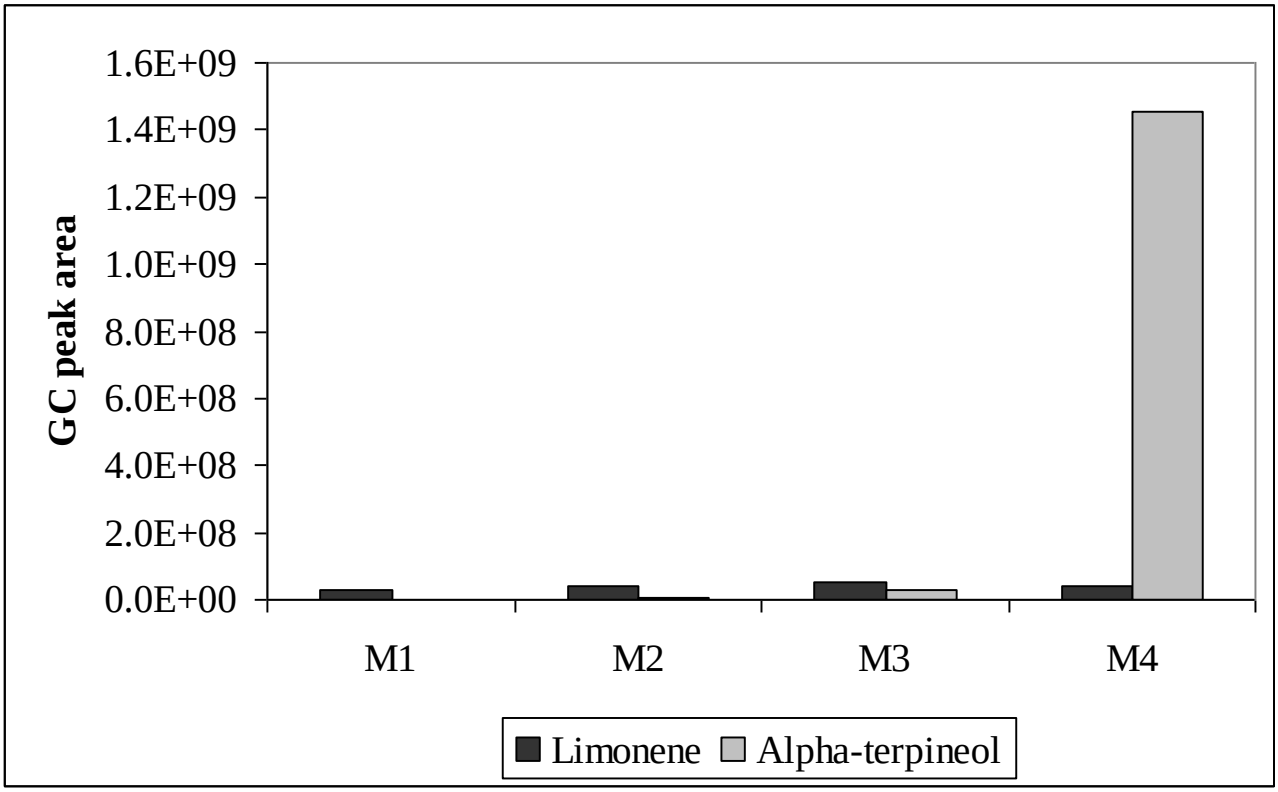

Figure 2. GC peak area of limonene and α-terpineol at the end of the batch anaerobic digestion of untreated and biologically treated OP2 in co-digestion with cow manure, mixtures M1 to M4 (see mixture compositions in Table 2).

The EE was equally as efficient at removing limonene at both ambient temperature and 40ºC. This is in line with the fact that the liquid-liquid equilibrium of the ternary mixture water-limonene-ethanol is independent of the temperature in the range of temperatures used in this work (Cháfer *et al.*, 2004). However, temperature could have an effect on the extraction efficiency of the minor components. This would also explain the similar methane yield increments observed with SD, despite the lower efficiency at limonene removal; since SD is carried out at temperatures higher than those used in the EE, the removal of minor components could have been improved. This could be explained by the similarity of the boiling points of limonene and some of the most abundant minor components of CEO. The boiling point of limonene is 175.5ºC -176ºC, and the boiling points of the most abundant of the compounds mentioned above are similar: 167ºC for myrcene, 156ºC for α-pinene, 166ºC for β-pinene, 158.5ºC for comphene and 171ºC for n-octanal (data from PubChem Compound Database, NCBI).

3.5 Energy balances

The thermal energy required for the pretreatments was estimated and compared with the thermal energy that could potentially be recovered from the methane generated from the treated OP. The results are displayed in Figure 3. The energy required for the pretreatment was higher than that potentially produced by the methane for all SD treatments. The opposite was the case for EE. The reason for this is the large amounts of energy necessary to generate the steam for SD, compared with the energy necessary to evaporate the residual alcohol in the case of EE.

From the point of view of OP valorization, in terms of energy production, the most interesting treatments are BT (no thermal energy required) and EE. The limonene recovery achieved with EE could improve the profitability of the whole process.

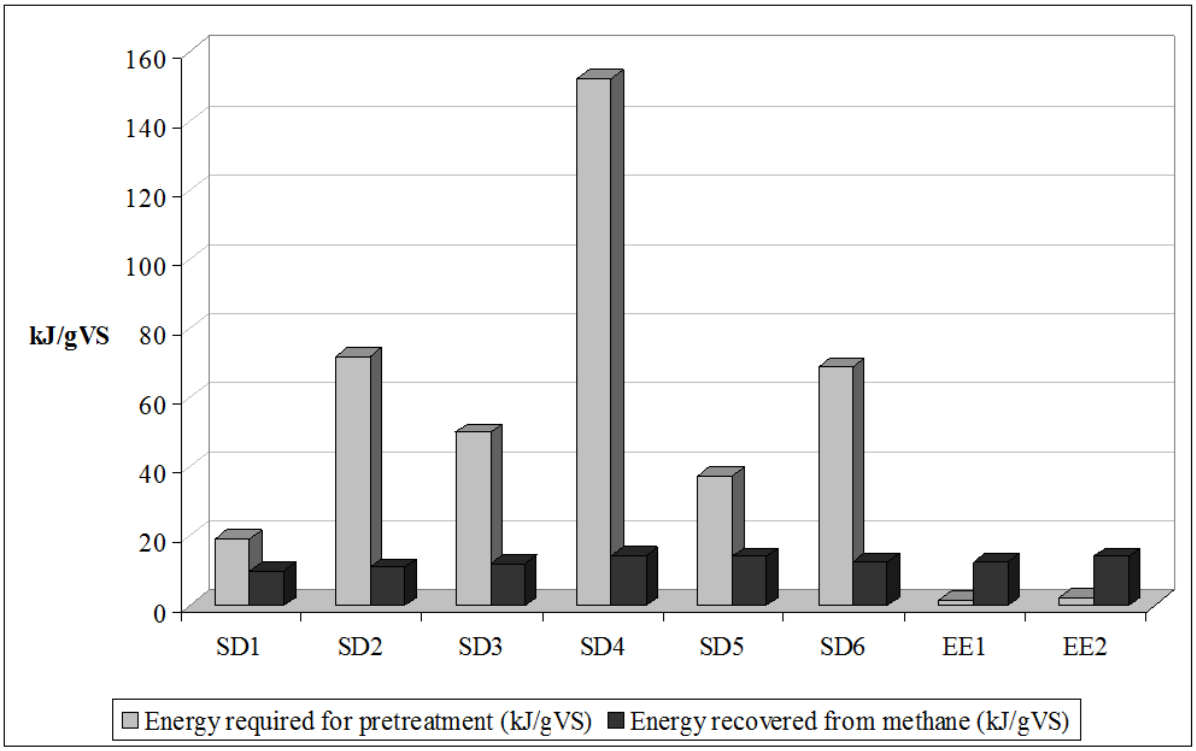

Figure 3. Thermal energy required for the pretreatments and potentially recovered from the methane generated with the pre-treated orange peel.

4 Conclusions

The three pretreatments applied to OP reduced the limonene concentration. The most efficient were EE, followed by SD and BT.

BT did not improve the methane yield. *Penicillium* is able to degrade limonene, but during the treatment α-terpineol can be produced, which exhibits strong inhibition.

SD and EE resulted in improved methane potential and production rate.

Favourable energy balance was obtained for BT and EE. Moreover, recovery of added value products can be achieved by EE, which could improve the profitability of the whole process.

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