Comparison between *in situ* dry matter degradation and *in vitro* gas production of tannin-containing leaves from four tree species

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Abstract

Dry matter (DM) degradation of *Glycrrhiza glabra* L, *Arbutus andrachne*, *Juniperus communis*, and *Pistica lentiscus* was determined using two different techniques: (i) the *in vitro* gas production and (ii) the *in situ* nylon bag degradability technique. Samples were incubated *in situ* and *in vitro* for 3, 6, 12, 24, 48, 72 and 96 h. *In situ* and *in vitro* DM degradation kinetics were described using the equation $y = a + b (1 - e^{-ct})$. At all incubation times except 3 and 72 h the cumulative gas production of *J. communis* was significantly lower than that of *G. glabra*, *A. andrachne* and *P. lentiscus*. At 3, 6 and 12 h incubation times the DM disappearance of *J. communis* was only significantly lower than that of *P. lentiscus.* At 24 and 48 h incubation times DM disappearance of *J. communis* was significantly lower than that of *A. andrachne* and *P. lentiscus.* There were significant relationships between *in vitro* gas production and *in situ* DM disappearance at 24 h and 96 h incubation times. The gas productions at 24 and 96 h incubation explained 51.2 and 52.4% of variation of DM disappearance, respectively. Gas production from the insoluble fraction (b) alone explained 66.4% of the variation of effective DM degradability (EDMD). The inclusion of gas production from quickly soluble fraction (a) and rate constant (c) of gas production in the regression equation did not improve the accuracy of predicting EDMD. It was concluded that *in situ* DM disappearance parameters of tannin-containing tree leaves such as used in this present study may be predicted from *in vitro* gas production parameters.

Keywords: Tree leaves, *in situ* dry matter degradation, *in vitro* gas production, tannin
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Introduction

The use of browse species as fodder for ruminant animals is becoming important in many parts of the world. The presence of tannins and other phenolic compounds in a large number of nutritionally important shrubs and tree leaves hampers their utilization as animal feed (Tolera *et al*., 1997). However, the information about the fermentation kinetics of tannin containing tree leaves is limited.

The rate and extent of fermentation of dry matter (DM) in the rumen are very important determinants for the nutrients absorbed by ruminants. The nylon bag technique has been used for many years to provide an estimate of both rate and extent of DM degradation of forages *in situ* (Mehrez & Ørskov, 1977). On the other hand, Menke *et al*. (1979) and Menke & Steingass (1988) developed the *in vitro* gas production technique to evaluate the nutritive value of forages and estimate the rate and extent of DM degradation indirectly using the gas production (CO_2) during fermentation. The *in situ* nylon bag and *in vitro* gas production technique are well correlated with animal performance (Ørskov, 1989), food intake (Blummel & Ørskov, 1993), microbial protein synthesis (Krishnamoorthy *et al*., 1991) and *in vivo* digestibility (Khazaal *et al*., 1993). More recently researchers have been investigating the relationship between fermentation kinetics of forages obtained by the *in situ* nylon bag technique and the *in vitro* gas production technique (Blummel & Ørskov, 1993; Khazaal *et al*., 1993; Dewhurst *et al*., 1995).

The aim of this study was to (I) determine fermentation kinetics of tannin containing tree leaves using the *in vitro* gas production and *in situ* nylon bag technique and (II) to determine whether it is possible to predict *in situ* DM degradability with the *in vitro* gas production technique.

Materials and Methods

Leaves from *Glycrrhiza glabra* L, *Arbutus andrachne*, *Juniperus communis* and *Pistica lentiscus* were harvested in the dry season (August, September and October) in the vicinity of the city, Kahramanmaras, in

the south of Turkey. The area is located at an altitude of 630 m above sea level. The mean annual rainfall and temperature are 857.5 mm and 16.2 °C, respectively. Leaves were hand harvested from at least 10 different trees, then pooled and oven dried at 60 °C for 48 h (Abdulrazak *et al*., 2000).

All chemical analyses were carried out in triplicate. Dry matter was determined by drying the samples at 105 °C overnight and ash by igniting the samples in a muffle furnace at 525 °C for 8 h. Nitrogen (N) content was measured by the Kjeldahl method (AOAC, 1990). Crude protein was calculated as N x 6.25. Concentrations of neutral detergent fibre (NDF) and acid detergent fibre (ADF) of leave samples were determined by the method of Van Soest *et al*. (1991). Total condensed tannin, bound condensed tannin and soluble condensed tannin were determined by the butanol-HCl method as described by Makkar *et al*. (1995). Mimosa tannin (MT; Hodgson, England) was used as an external standard.

Forage samples, milled through a 1 mm sieve, were incubated in rumen fluid in calibrated glass syringes, following the procedures of Menke & Steingass (1988). Rumen fluid was obtained from three fistulated sheep fed twice daily on a diet containing lucerne hay (60%) and concentrate (40%). Dry samples (0.2 g) were weighed in triplicate into calibrated glass syringes of 100 mL. The syringes were prewarmed at 39 °C before the injection of 30 mL rumen fluid-buffer mixture into each syringe followed by incubation in a water bath at 39 °C. Readings of gas production were recorded before incubation (0) and 3, 6, 12, 24, 48, 72 and 96 h after incubation. Total gas values were corrected for blank incubation. Cumulative gas production data were fitted to the exponential equation: $p = a + b (1-e^{-ct})$ (Ørskov & McDonald, 1979):
where p is the gas production at time t; a is the gas production from the immediately soluble fraction (mL), b is the gas production from the insoluble fraction (mL), c is the gas production rate constant, a + b = the potential gas production (mL), t = incubation time (h).

The *in situ* DM degradation analysis was carried out according to the procedure described by Mehrez & Ørskov (1977). Five gram samples dried and milled through a 3 mm sieve were weighed into nylon bags and incubated for 3, 6, 12, 24, 48, 72 and 96 h in three rumen fistulated sheep. A completely randomized block design was used. The sheep were fed twice a day on a 60% lucerne hay and 40% concentrate diet. After removal, the nylon bags were thoroughly washed with running cold water until no further coloured liquid could be extruded, and dried at 60 °C for 48 h. Dry matter losses for each incubation time were determined. The DM degradation data were fitted to the exponential equation $p = a + b (1-e^{-ct})$ (Ørskov & McDonald (1979): where p is DM disappearance in rumen at time t, a is the rapidly soluble fraction, b is the insoluble but fermentable fraction, c = the constant rate of degradation of b (percentage per h). Effective DM degradability (EDMD) was calculated applying the equation of Ørskov & McDonald (1979): EDMD = a + (bc / (c+k)), where k is the rumen outflow rate of 2% per h, which is at the maintenance level.

Analysis of variance (ANOVA) was carried out for *in vitro* gas production, *in situ* DM disappearance and estimated parameters using General Linear Model (GLM) of Statistica for Windows (1993). The experiment was not replicated at field harvesting. Therefore tree species were not compared in terms of chemical composition. A complete randomized block design for *in situ* DM degradation was used. Because of the differences between the sheep, each sheep was considered as a block and the tree species were fixed factors in the linear model.

Significant differences between individual means were identified using the Tukey's Multiple Range Test (Pearse & Hartley, 1966). Mean differences were considered significant at $P < 0.05$. Standard errors of means were calculated from the residual mean square in the analysis of variance. A simple correlation analysis was used to establish the relationship between chemical composition, *in situ* DM degradation and *in vitro* gas production.

Results

The proximate composition and condensed tannin concentration of the tree leaves are presented in Table 1. Generally there were considerable variations between tree and shrub leaves in terms of chemical compositions. The CP concentration of tree leaves ranged from 72.5 to 125.9 g/kg DM. The ash level of leaves ranged from 65.9 to 78.9 g/kg DM. The NDF and ADF levels ranged from 343.7 to 571.7 g/kg DM and from 251.7 to 333.5 g/kg DM, respectively.

Data on gas production during the fermentation period are given in Table 2. The cumulative volume of gas production increased with increasing time of incubation. Gas produced after 96 h incubation ranged between 61.84 and 73.55 mL per 0.2 g of substrate.

At all incubation times the cumulative gas production of *J. communis* was lower (P < 0.001) than that of *G. glabra, A. andrachne* and *P. lentiscus* except at the 3 and 72 h incubations. Therefore, the estimated parameters (constant a, b and a+b) of *J. communis* are lower (P < 0.001) than those of *G. glabra, A. andrachne* and *P. lentiscus* whereas the gas production rate (c) of b for *J. communis* was lower (P < 0.01) than that of *G. glabra* and *A. andrachne*.

Table 1 Mean chemical composition (g/kg DM) of leaves from different trees

	Tree species			
	Glycyrrhiza glabra	*Arbutus andrachne*	*Juniperus communis*	*Pistica lentiscus*
Dry matter	929.1	959.3	948.2	956.1
Ash	78.9	65.9	77.0	72.0
Crude protein	125.9	72.5	107.3	95.0
Neutral detergent fibre	343.7	348.7	571.7	431.7
Acid detergent fibre	251.5	319.7	333.5	305.8
Total condensed tannin	126.6	120.4	197.0	156.9
Bound condensed tannin	85.5	30.0	170.0	23.5
Soluble condensed tannin	41.0	93.0	26.9	130.9

Table 2 *In vitro* gas production (mL) and estimated parameters of leaves from different trees when incubated with buffered rumen liquid

	Incubation times (h)						
Tree species	3	6	12	24	48	72	96
G. glabra	21.8^{c}	35.2^{c}	45.2^{b}	55.0^{b}	64.9^{b}	69.9^{b}	73.6^{b}
A. andrachne	20.3^{bc}	30.7^{bc}	41.9^{b}	52.9^{b}	59.9^{a}	65.3^{a}	69.8^{b}
J. communis	15.1^{a}	25.0^{a}	36.3^{a}	46.6^{a}	56.5^{a}	61.4^{a}	61.8^{a}
P. lentiscus	18.0^{ab}	29.7^{b}	42.7^{b}	55.00^{b}	65.4^{b}	69.9^{b}	72.3^{b}
s.e.m.	0.85	1.03	1.16	0.61	0.96	1.02	1.07
Sig.	***	***	***	***	***	***	***
	Estimated parameters						
	c	a	b	a+b			
G. glabra	0.087^{b}	3.83^{b}	64.9^{c}	68.8^{c}			
A. andrachne	0.084^{b}	3.37^{b}	61.4^{b}	64.8^{b}			
J. communis	0.070^{a}	2.21^{b}	57.9^{a}	60.34^{a}			
P. lentiscus	0.075^{ab}	3.13^{ab}	66.8^{c}	70.00^{c}			
s.e.m.	0.003	0.240	0.64	0.76			
Sig.	**	**	***	***			

$^{a\ b\ c}$ Column means with common superscripts do not differ (P > 0.05); s.e.m. - standard error mean; Sig. - significance level; c - gas production rate (%); a - gas production (mL) from quickly soluble fraction; b - gas production (mL) from the insoluble fraction; a+b - potential gas production (mL)
*** P < 0.001; ** P < 0.01

Data of DM disappearance from nylon bags during the fermentation periods are given in Table 3. The disappearance of DM increased with increasing time of incubation. The DM disappearance after 96 h incubation ranged between 70.7 and 77.6%.

After 3, 6 and 12 h incubation, the DM disappearance of *J. communis* was lower (P < 0.05) only to that of *P. lentiscus.* After 24 and 48 h incubation the DM disappearance of *J. communis* was lower (P < 0.01) than that of *Arbutus andrachne* and *Pistica lentiscus.* After 96 h incubation the DM disappearance of *J. communis* was lower (P < 0.001) than that of *G. glabra*, *A. andrachne* and *P. lentiscus.* The rate of DM disappearance of *P. lentiscus* was higher (P < 0.05) than the others. There were no significant (P > 0.05) differences between tree leaves in terms of the quickly soluble fraction (a) whereas fraction b of *A. andrachne* was higher (P < 0.05) than the others. The EDMD of *J. communis* was lower (P < 0.001) than that of *A. andrachne* and *P. lentiscus.*

Table 3 *In situ* dry matter disappearance and estimated parameters of leaves from different trees when incubated within rumen

	Incubation times (h)						
Tree species	3	6	12	24	48	72	96
G. glabra	26.7^{ab}	35.7^{a}	47.7^{ab}	56.6^{a}	66.5^{ab}	71.3^{ab}	75.1^{b}
A. andrachne	27.5^{b}	38.6^{ab}	45.9^{ab}	59.2^{ab}	69.4^{b}	73.0^{ab}	77.6^{b}
J. communis	25.4^{a}	34.3^{a}	43.2^{a}	53.9^{a}	63.6^{a}	69.2^{a}	70.7^{a}
P. lentiscus	29.3^{c}	41.9^{b}	54.0^{b}	63.4^{b}	70.1^{b}	74.6^{b}	77.2^{b}
s.e.m.	0.33	1.07	2.09	1.22	0.86	0.99	0.62
Sig.	***	**	*	**	**	**	***
	Estimated parameters						
	c		a		b		EDMD
G. glabra	0.038^{a}		23.4		52.2^{ab}		57.6^{ab}
A. andrachne	0.035^{a}		24.3		56.6^{b}		59.2^{bc}
J. communis	0.034^{a}		23.1		50.0^{a}		54.8^{a}
P. lentiscus	0.048^{b}		25.6		51.3^{a}		61.7^{c}
s.e.m.	0.003		0.61		0.93		0.68
Sig.	*		NS		*		***

$^{a\ b\ c}$ Column means with common superscripts do not differ (P > 0.05); s.e.m. - standard error mean; Sig. - significance level; c - the constant rate of degradation of b (percentage per h); a - water soluble fraction (%); b - insoluble but fermentable fraction (%); EDMD - effective dry matter degradability (%)
NS - non significant; *** P < 0.001; ** P < 0.01; * P < 0.05

The correlation coefficients (r) of the relationship between the chemical composition and gas production or *in situ* DM degradation parameters from different tree leaves are given in Table 4 and 5.

Table 4 Correlation coefficients (r) of the relationship between the chemical composition and gas production or estimated parameters from different tree leaves

	Chemical constituents					
In vitro	CP	ADF	NDF	TCT	BCT	SCT
3	0.138	-0.721**	-0.899***	-0.630*	-0.503	0.160
6	0.339	-0.751**	-0.848***	-0.594*	-0.438	0.101
12	0.153	-0.812***	-0.835***	-0.641*	-0.636*	0.319
24	0.020	-0.643*	-0.832***	-0.387	-0.786**	0.527
48	0.273	-0.691*	-0.609*	-0.399	-0.600*	0.467
72	0.259	-0.659*	-0.655*	-0.589*	-0.592*	0.449
96	0.148	-0.719**	-0.830***	-0.589*	-0.717*	0.473
c	0.050	-0.719**	-0.816***	-0.732**	-0.406^{S}	-0.038
a	0.180	-0.505^{NS}	-0.830***	-0.621*	-0.505	0.165
b	0.151	-0.595*	-0.590*	-0.409	-0.689*	0.572
a+b	0.179	-0.602*	-0.654*	-0.461	-0.690*	0.532

CP - crude protein (g/kg DM); ADF - acid detergent fibre (g/kg DM); NDF - neutral detergent fibre (g/kg DM); TCT - total condensed tannin (g/kg DM); BCT - bound condensed tannin (g/kg DM); SCT - soluble condensed tannin (g/kg DM); c - gas production rate (%); a - gas production (mL) from quickly soluble fraction; b - gas production (mL) from the insoluble fraction; a+b - potential gas production (mL)
*** P < 0.001; ** P < 0.01; * P < 0.05

The regression equations describing the relationship between *in situ* DM degradation and *in vitro* gas production are given in Table 6. There were no significant relationships between gas production and DM disappearance at 3, 6, 12, 48 and 72 h of incubation whereas there were significant relationships at 24 h and 96 h incubation times. The gas productions after 24 and 96 h incubation explained 51.2 and 52.4% of variation of DM disappearance, respectively.

Table 5 Correlation coefficients (r) of the relationship between the chemical composition and dry matter disappearance or estimated parameters from different tree leaves

	Chemical constituents					
In situ	CP	ADF	NDF	TCT	BCT	SCT
3	-0.214	-0.008	-0.379	-0.247	-0.718**	0.747**
6	-0.356	-0.002	-0.320	-0.327	-0.741**	0.714**
12	-0.009	-0.308	-0.288	-0.274	-0.588*	0.535
24	-0.373	-0.143	-0.389	-0.192	-0.776**	0.876***
48	-0.411	-0.075	-0.594*	-0.357	-0.826***	0.819***
72	-0.410	-0.178	-0.469	-0.145	-0.732**	0.866***
96	-0.485	0.336	-0.759**	-0.521	-0.897***	0.790**
c	0.077	-0.090	-0.105	-0.420	-0.420	0.446^{S}
a	-0.388	-0.091	-0.219	-0.133	-0.594*	0.678**
b	-0.571	-0.650*	-0.017	-0.419	-0.513	0.383
EDMD	-0.344	-0.203	-0.516	-0.317	-0.837***	0.854***

CP - crude protein; ADF - acid detergent fibre; NDF - neutral detergent fibre; TCT - total condensed tannin; BCT - bound condensed tannin; SCT - soluble condensed tannin; c - the constant rate of degradation of b (percentage per h); a - water soluble fraction; b - insoluble but fermentable fraction; EDMD - effective dry matter degradability
*** P < 0.001; ** P < 0.01; * P < 0.05

Table 6 Prediction of *in situ* dry matter (DM) disappearance and estimated parameters from *in vitro* gas production and estimated parameters

Y	Equation and factors used	R^2	RSD	Probability
In situ				
3	$Y=23.6 + 0.195gas_{3h}$	11.4	1.682	> 0.05
6	$Y=31.6 + 0.199gas_{6h}$	5.2	3.619	> 0.05
12	$Y= 17.6 + 0.736gas_{12h}$	31.3	4.366	> 0.05
24	$Y= 17.9 + 0.772\ gas_{24h}$	51.2	2.730	< 0.01
48	$Y= 41.7 + 0.416gas_{48h}$	33.4	2.520	< 0.05
72	$Y= 46.8 + 0.379\ gas_{72h}$	38.1	2.020	< 0.05
96	$Y=44.1 + 0.447gas_{96h}$	52.4	2.220	< 0.01
c_{is}	$Y= 0.0488 – 0.118c_{gas}$	1.9	0.007	> 0.05
a_{is}	$Y= 23.9 + 0.058a_{gas}$	0.1	1.502	> 0.05
b_{is}	$Y= 52 + 0.007b_{gas}$	0.0	2.990	> 0.05
EDMD	$Y= 52.3 + 1.90\ a_{gas}$	65.7	1.830	< 0.01
EDMD	$Y= 19.4 + 0.620b_{gas}$	66.4	1.921	< 0.05
EDMD	$Y= 52.7 + 70c_{gas}$	4.3	2.898	> 0.05
EDMD	$Y= 18.6 - 0.190a_{gas} + 0.64b_{gas}$	65.7	1.830	< 0.01
EDMD	$Y= 20.5 - 0.02a_{gas} + 0.645b_{gas} -34.2c_{gas}$	66.4	1.921	< 0.01
EDMD	$Y= 23.5 + 0.580\ (a+b)_{gas} - 43.6c_{gas}$	63.7	1.881	< 0.01
EDMD	$Y= 20.4 + 0.647\ b_{gas} – 33.6c_{gas}$	66.4	1.810	< 0.01
EDMD	$Y= 22.4 + 0.545\ (a+b)gas$	62.3	1.818	< 0.01

c_{is} - rate of DM degradation; a_{is} - quickly soluble fraction; b_{is} - insoluble but fermentable fraction; EDMD - effective DM degradability; c_{gas} - gas production rate; a_{gas} - gas production (mL) from quickly soluble fraction; b_{gas} - gas production (mL) from the insoluble fraction; $(a+b)_{gas}$ - potential gas production (mL)
Sig. - significance level

Discussion

The average chemical composition of the studied samples is consistent with published results, despite differences in location and growth conditions. The chemical composition of *P. lentiscus* was similar to that reported by Decandia *et al*. (2000). The TCT concentration of *P. lentiscus* was lower than that reported by Silanikove *et al*. (1996) and Decandia *et al*. (2000).

Gas production and most of the estimated parameters showed a negative correlation with NDF, ADF, TCT and BCT. This is consistent with findings of Khazaal & Ørskov (1994), Tolera *et al*. (1997) and Abdulrazak *et al*. (2002). *In situ* DM disappearance after 48 and 96 h was negatively correlated with NDF. *In situ* DM disappearance at all incubation times was negatively correlated with BCT, but positively correlated with SCT except after 12 h incubation. The NDF and ADF concentrations of tree leaves did not show any significant correlation with DM disappearance and estimated parameters. This result is in agreement with findings of Tolera *et al*. (1997).

The gas productions were well correlated with DM disappearance after 24 and 96 h incubation. This is in agreement with findings of Sileshi *et al*. (1996) but not with that of Blümmel & Ørskov (1993) who did not find any correlation between these parameters in barley and wheat straw. Beuvinik *et al*. (1993) and Beuvink & Kogutk (1993) suggested that the relationship between these parameters varies with type of forage. On the other hand there was no significant relationship between the estimated parameters of gas production and DM degradation of tree leaves in this experiment. This is in agreement with the findings of Beuvink & Kogutk, (1993) and Blümmel & Ørskov (1993) who showed that there was no significant correlation between rate of gas production and rate of DM disappearance. On the other hand, Sileshi *et al.* (1996) found a significant relationship between these parameters. There is no obvious explanation for these anomalies. Differences between the conclusions drawn by different authors may be due to a number of factors such as methodology, the substrates and the physical form used.

Gas production from the insoluble fraction (b) alone explained 66.4% of the variation of EDMD. The inclusion of gas production from the quickly soluble fraction (a) and the rate constant (c) of gas production in the regression equation did not improve the accuracy of predicting EDMD.

The EDMD of tree leaves was highly correlated ($r = 0.790$, $P < 0.001$) to potential *in vitro* gas production. This is in agreement with Blümmel & Ørskov (1993), Khazaal *et al.* (1993) and Sileshi *et al.* (1996). The highly significant relationship between gas production and DM disappearance after 24 and 96 h incubation suggests that either method could be used to estimate the nutritive value of such tree leaves. Khazaal *et al.* (1994) reported that the *in situ* method should be used with caution when estimating the nutritive value of feed containing high levels of phenolic compounds. The potential negative effect of phenolic compounds on microbial fermentation is unlikely to be detected by the *in situ* method. In this regard *in vitro* methods are more reliable in detecting inhibitory compounds in feeds. The *in vitro* gas production method is a closed system with a limited supply of rumen liquor. Any anti-nutritive compound is likely to affect the activity of the rumen microbes. On the other hand, the *in situ* method is associated with a dilution effect which results from an open system with a wider rumen environment and copious supply of rumen fluid to nylon bag content (Apori *et al*., 1998). This is why the DM disappearance and some estimated parameters showed a positive correlation with SCT in this experiment (Table 5).

A low relationship (especially at 3, 6, 12, 48 and 72 h incubation times) between gas production and DM disappearance may be due to the interference of tannin in the leaves. The relationship between the two techniques might be increased when tannin-containing tree leaves were incubated in the presence of polyethylene glycol (PEG) since there is a significant negative correlation between gas production and TCT or BCT contents. Some studies clearly showed that PEG supplementation increased gas production (Getachew *et al*., 2001; Getachew *et al*., 2002; Seresinhe & Iben, 2003). However, tree leaves did not give the same response to PEG supplementation, possibly due to differences in the chemical composition of tannins in tree leaves. Although the increase in the gas production of *Acacia cyanophylla* was 10.3 mL, the increase in the gas production of *C. calothyrsus* was 22 mL when they were incubated in the presence of PEG (77 mg) (Getachew *et al.*, 2001). More studies are required to test the effect of PEG supplementation on the relationship between *in vitro* gas and *in situ* DM degradation.

This study revealed a general problem of overestimation of degradability by the nylon bag technique. Overestimation is especially noticeable at short incubation times. The ability to use *in vitro* gas production methods to study the kinetics of degradation of tannin containing forage instead of the *in situ* technique would have advantages, including avoiding the error associated with loss of small particles through the pores of the nylon bag.

Conclusion

There were significant relationships between parameters obtained using two techniques; the *in situ* digestion and *in vitro* gas production. Therefore, it was concluded that DM disappearance parameters of

tannin-containing tree leaves may be predicted from *in vitro* gas production parameters. The relationship between parameters might be increased when tree samples were incubated in the presence of PEG. Therefore, more investigations are required to see the effect of PEG supplementation on the improvement of the relationship between *in vitro* gas and *in situ* DM degradation.

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