Spanish Journal of Agricultural Research
13(2), e06SC01, 6 pages (2015)
eISSN: 2171-9292
http://dx.doi.org/10.5424/sjar/2015132-6463
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)

SHORT COMMUNICATION

OPEN ACCESS

The effect of different inclusion levels of polyethylene glycol as a silage additive on ensilage characteristics of pomegranate peel and *in vitro* rumen fermentation

Ali Hatami[1], Daryoush Alipour[1*], Fardin Hozhabri[2] and Meisam Tabatabaei[3,4]

[1] Bu-Ali Sina University, Faculty of Agriculture, Department of Animal Science. Hamedan, Iran [2] Razi University, Faculty of Agriculture, Department of Animal Science. Kermanshah, Iran [3] Biofuel Research Team (BR Team), Karaj, Iran [4] Agricultural Biotechnology Research Institute of Iran (ABRII), Microbial Biotechnology and Biosafety Department. Karaj, Iran

Abstract

This study was conducted to evaluate the effects of ensiling pomegranate peel (PP) with different levels of polyethylene glycol (PEG) on its chemical composition, tannin content, *in vitro* gas production and fermentation characteristics. Fresh PP was chopped and ensiled in mini silos made of polyvinyl chloride tubing. Five levels of PEG were studied: 0 (control), 5, 10, 15, and 20% of fresh PP (dry matter basis). Total phenolics, total tannins, crude ash, crude protein, neutral detergent fiber and acid detergent fiber content and pH decreased with increasing PEG levels, whereas dry matter and non-fiber carbohydrates content, non-tannin phenols, lactic acid and ammonia concentrations and buffering capacity increased. The water soluble carbohydrates and ether extract concentrations were not influenced by the addition of PEG. The partitioning factor and efficiency of microbial biomass production were quadratically decreased (p=0.020 and p=0.032, respectively) as PEG inclusion increased, but the *in vitro* apparent dry matter disappearance did not differ among treatments. Compared to control, the *in vitro* true disappearance and *in vitro* fiber digestibility had a tendency to be higher in silages treated with PEG (p=0.081 and p=0.069, respectively). The metabolizable energy content and total volatile fatty acids concentration increased quadratically by PEG inclusion. The asymptotic gas production and rate of gas production were higher in PEG-treated silages. Overall, ensiling PP with PEG can improve the fermentation characteristics of this by-product.

Additional key words: *Punica granatum;* agro-industrial by-products; tannin; microbial biomass; lactic acid; partitioning factor

Abbreviations used: ADF (acid detergent fiber); CP (crude protein); DM (dry matter); dNDF (*in vitro* fiber digestibility); EE (ether extract); EMBP (efficiency of microbial biomass production); GP (gas production); ivTD (*in vitro* true disappearance); L (linear); MBP (microbial biomass production); ME (metabolizable energy); NDF (neutral detergent fiber); NFC (non-fibrous carbohydrates); PEG (polyethylene glycol); PF (partitioning factor); PP (pomegranate peel); Q (quadratic); TVFA (total volatile fatty acids).

Citation: Hatami, A.; Alipour, D.; Hozhabri, F.; Tabatabaei, M. (2015). Short communication: The effect of different inclusion levels of polyethylene glycol as a silage additive on ensilage characteristics of pomegranate peel and *in vitro* rumen fermentation. Spanish Journal of Agricultural Research, Volume 13, Issue 2, e06SC01, 6 pages. http://dx.doi.org/10.5424/sjar/2015132-6463

Received: 30 Jun 2014. **Accepted:** 06 Mar 2015

Funding: The authors received no specific funding for this work

Competing interests: The authors have declared that no competing interests exist.

Correspondence should be addressed to Daryoush Alipour: alipourd@basu.ac.ir; daryoush.alipour@gmail.com

Pomegranate (*Punica granatum*), native to Persia (Iran), is an edible fruit cultivated in subtropical and tropical regions around the world with different microclimatic zones such as in the United States, Turkey, Egypt, Italy, India, Chile and Spain. Pomegranate juice has nutritional and medical benefits and, due to the increasing consumer awareness of its potential health benefits, the global production and consumption of pomegranate has greatly expanded in recent years. The edible parts of pomegranate are used extensively in the form of juice, concentrate, canned beverage, jam and jelly. Pomegranate peel (PP) obtained after juice extraction accounts for about 50% of the weight of pomegranate fruit and its high moisture and nutrient content, as well as its phenolics, can cause environmental pollution. The presence of phenolics in the waste waters considerably increases biochemical and chemical oxygen demands, with detrimental effects on the flora and fauna of discharge zones. In solid residues high levels of phenolic compounds are problematic because of their inhibition of germination properties (Negro *et al.,* 2003).

A number of studies have suggested the application of PP in animal nutrition. However, its high content of tannin has been reported to cause some problems when fed to animals (Makkar, 2003; Shabtay *et al.,* 2008). To ameliorate the negative effects of dietary tannins, several methods such as using polyethylene glycol (PEG) and ensiling are proposed (Makkar, 2003). Since PP is produced within a short time period (during the harvest season), ensiling seems to be an efficient way to inactivate PP tannins. Little information is available on simultaneous application of PEG and ensiling in tannin-containing by-products such as PP. Therefore, the aim of the current experiment was to assess the effect of different inclusion levels of PEG on ensilage characteristics and *in vitro* gas production (GP) parameters of PP.

Fresh PP was collected from the Sahar factory (Hamadan, Iran) and chopped to a cut length of 20-30 mm using a forage chopper. The chopped PP (32.4% dry matter (DM)) was then ensiled in mini silos made of polyvinyl chloride tubing (15 cm diameter and 70 cm height; capacity 12 kg) and the filled silos were sealed with plastic lids. Five levels of PEG (MW6000; Shazand Petrochemical CO, Arak, Iran) were added to fresh PP before ensiling: 0 (control), 5, 10, 15, and 20% of fresh PP (DM basis). Four replications were used for each treatment. All mini-silos were kept in laboratory for 70 days.

After opening the mini-silos, subsamples were taken and stored at –20 °C until further analysis. The DM, crude protein (CP), ether extract (EE), crude ash (AOAC, 1990), neutral detergent fiber (NDF; without a heat stable amylase and ash included) and acid detergent fiber (ADF) content were measured (Van Soest *et al.,* 1991). Non-fibrous carbohydrates (NFC) content was calculated using the following equation (NRC, 2001):

$$NFC\% = 100 - (\%\ NDF + \%\ CP + \%\ EE + \%Ash)$$

Total phenolics, non-tannin phenols and total tannins were determined as described by Makkar (2000). Total volatile fatty acids (TVFA) content of sample extracts were determined by steam distillation (Barnett & Reid, 1957), lactic acid concentration as described by Taylor (1996) and water-soluble carbohydrates content using the water extraction–anthrone method (MAFF, 1982). The pH of silages was analyzed according to Faithfull (2002) using a portable digital pH meter (pH315i, WTW 82362 Weilheim, Germany). Ammonia was measured in the extract by the phenol-hypochlorite assay (Broderick & Kang, 1980) and the buffering capacity of silage was determined according to Moharrery (2007).

The *in vitro* gas test method was performed according to Menke & Steingass (1988). Rumen liquor samples were obtained from three rumen-canulated sheep fed twice daily a diet containing alfalfa hay (650 g/kg) plus concentrate mixture (350 g/kg) prior to their morning feeding. Samples were pooled and placed in a prewarmed CO_2-filled flask and transported immediately to the laboratory. Rumen liquor was mixed, squeezed through four layers of cheesecloth and added to the buffer solution (1:2, v/v), which was kept in a water bath at 30 °C with CO_2 saturation. Ensiled samples (200mg) were weighed in triplicate and placed in glass syringes to which 30 ml of buffered-rumen liquor was dispensed. Then, the glass syringes were immediately placed into a thermostatically-controlled water bath (39 ± 0.5 °C). Three syringes containing only buffered-rumen fluid (no silage sample) were also included in the experiment and the mean GP value for these syringes was used as the blank value.

Cumulative GP was recorded after incubation for 0, 2, 4, 6, 8, 12, 16, 20, 24, 48, 72, 96, 120 and 144 h. All incubation experiments were done in three runs. The kinetic parameters of GP were fitted using the exponential model proposed by France *et al*. (2000) as follows:

$$G = A\ (1 - e^{-ct})$$

where G (mL) denotes the cumulative GP at time t; A (mL/g DM) is the asymptotic GP, c (h^{-1}) is the fractional rate of GP. Since the lag time was equal to zero in all cases, it was removed from original equation.

In separate runs of GP, fermentation parameters and substrate digestibility were measured in syringes containing 500 mg substrates and 40 mL buffered rumen fluid. Gas production after 24 hours of incubation (GP_{24}), the *in vitro* apparent dry matter disappearance, *in vitro* true dry matter disappearance (ivTD), *in vitro* fiber digestibility (dNDF), partitioning factor (PF) and ammonia concentration in acidified rumen fluid (1 mL 6 N HCl per 15 mL of filtrate) were measured as described by Taghavi-Nezhad *et al.* (2011).

In vitro microbial biomass production (MBP) and efficiency of microbial biomass production (EMBP) were estimated from concomitant gas volume and truly disappeared dry matter (ivTD) measurements (Blümmel, 2000) as:

$$MBP = ivTD - (gas\ volume \times SF)$$

$$EMBP = \frac{ivTD - (gas\ volume \times SF)}{ivTD}$$

where SF is the stoichiometric factor, that has a value of 2.20 for forages.

The metabolizable energy (ME) content of silages was calculated using equation proposed by Menke *et al.* (1979).

The data were analyzed according to a completely randomized design using the GLM procedure of SAS (2004). Data of each of the three days within the same levels of PEG were averaged before statistical analysis. Mean values of each individual level of PEG (three replicates for each) were used as the experimental unit. The model used for analysis was:

$$Yij = \mu + \beta i + \varepsilon ij$$

where Yij is the value of each individual observation for the dependent variable, μ is the overall mean, βiis the effect of i level of PEG (i = 0, 5, 10, 15, 20) and εij is the random residual error. The effect of addition of PEG was evaluated with a contrast testing the difference between the control and all treatments receiving the PEG preparation (control *vs.* PEG). The effects of the dose of PEG added were assessed using orthogonal polynomial contrasts to test for linear (L), quadratic (Q) and cubic effects of the level of PEG. Probability levels less than $p < 0.05$ were considered significant, whereas $0.05 < p < 0.10$ was considered a trend.

The chemical composition of the different silages is presented in Table 1. The crude ash, CP, NDF and ADF concentrations decreased linearly ($p < 0.001$, $p = 0.001$, $p = 0.016$, and $p = 0.019$, respectively) with increasing PEG levels, whereas EE concentration was not influenced by the addition of PEG. The inclusion of PEG increased DM concentration as well as the NFC concentration. The amount of total phenolics and total tannins decreased linearly ($p = 0.004$ and $p = 0.007$, respectively), whereas a linear increment ($p = 0.028$) was seen in non-tannin phenols due to the addition of PEG. The lactic acid concentration and buffering capacity values increased, whereas pH value was lowered by including PEG in the PP silages (Table 2). The ammonia concentrations in the PEG-treated silages

Table 1. Chemical composition of pomegranate peel silages treated with different amounts of PEG

Levels of PEG	DM	CP	Ash	NDF	ADF	EE	NFC	TP	TT	NTP
0	327.9	51.0	48.4	300	173.7	19.7	580.8	191.7	162.4	29.3
5	342.1	50.4	48.2	304	156.4	18.1	579	180.7	149.7	31.0
10	341	50.1	46.7	300.3	164.4	20.0	582.8	165.7	131.2	34.5
15	366.6	49.8	41.6	290.3	157	20.5	597	157.0	122.5	34.5
20	378.4	48.6	39.9	274.9	147.6	18.5	616.3	164.9	132.1	32.8
SEM	2.649	0.437	1.053	7.484	4.824	2.076	7.549	7.22	7.47	1.29
p-values										
Linear	< 0.001	0.001	< 0.001	0.016	0.019	1.000	0.002	0.004	0.007	0.028
Quadratic	0.043	0.468	0.112	0.126	0.977	0.807	0.082	0.079	0.126	0.058
Cubic	0.860	0.393	0.171	0.924	0.133	0.385	0.978	0.333	0.393	0.423
Control *vs.* PEG	0.001	0.018	0.001	0.383	0.023	0.838	0.145	0.006	0.013	0.022

PEG: polyethylene glycol (% of dry matter); DM: dry matter (g/kg fresh weight), CP: crude protein (g/kg DM), NDF: neutral detergent fiber(g/kg DM), ADF: acid detergent fiber (g/kg DM), EE: ether extract (g/kg DM), NFC: non fiber carbohydrates (g/kg DM), TP: total phenols (g of tannic acid equivalents /kg DM), TT: total tannins (g of tannic acid equivalents /kg DM); NTP: non-tannin phenols (g of tannic acid equivalents /kg DM).

Table 2. Fermentation ensiling characteristics of pomegranate peel silages treated with different amounts of PEG

Levels of PEG	pH	Lactic acid (g/kg DM)	TVFA (meq/kg DM)	Ammonia (g/kg total N)	WSC (g/kg DM)	BC (meq/L)
0	3.69	42.7	125.8	97.7	24.62	12.73
5	3.60	48.3	132.1	103.1	23.33	15.58
10	3.59	48.1	132.8	107.1	21.81	16.06
15	3.56	53.0	131.6	103.3	21.62	15.75
20	3.61	53.1	133.0	103.7	24.62	15.47
SEM	0.030	0.52	3.47	3.65	1.515	0.2693
p-values						
Linear	0.040	<0.001	0.227	0.380	0.650	<0.001
Quadratic	0.036	0.005	0.383	0.262	0.146	<0.001
Cubic	0.605	0.517	0.469	0.594	0.905	0.013
Control *vs.* PEG	0.007	<0.001	0.112	0.089	0.632	<0.001

PEG: polyethylene glycol (% of dry matter); WSC: water soluble carbohydrates; TVFA: total volatile fatty acids; BC: buffering capacity.

Table 3. *In vitro* rumen fermentation parameters of pomegranate peel silages treated with different amounts of PEG

Levels of PEG	A	C	GP_{24}	ivAD	ivTD	dNDF	ME	TVFA	PF	EMBP	MBP	Ammonia
0	173.1	0.222	163.78	590	770.3	251	7.01	0.723	4.33	0.491	348.2	25.38
5	202.6	0.286	184.86	643.3	796.6	339.7	7.59	0.816	3.85	0.428	305.27	28.43
10	201.1	0.303	182.7	655.7	803.8	346.1	7.53	0.807	3.95	0.443	319.91	30.02
15	193.7	0.305	181.79	655.7	815.3	363.2	7.5	0.803	4.02	0.453	331.92	30.05
20	200.6	0.279	179.8	600.7	799.4	317	7.43	0.794	4.01	0.449	323.96	30.58
SEM	7.526	0.0226	4.526	38.5	13.7	35.1	0.123	0.0201	0.868	0.0121	10.294	0.4442
p-values												
Linear	0.11	0.122	0.070	0.792	0.137	0.220	0.084	0.070	0.111	0.166	0.517	<0.001
Quadratic	0.129	0.067	0.024	0.173	0.179	0.105	0.022	0.024	0.020	0.032	0.111	0.003
Cubic	0.115	0.785	0.152	0.912	0.855	0.871	0.157	0.152	0.034	0.039	0.038	0.173
Control *vs*. PEG	0.025	0.036	0.004	0.3081	0.081	0.069	0.005	0.004	0.003	0.006	0.035	0.002

PEG: polyethylene glycol (% of dry matter); A: potential gas production (mL/g DM); C: rate of gas production (h^{-1}); GP_{24}: gas production in 24 h (mL/g DM); ivAD: *in vitro* apparent dry matter disappearance (g/kg DM); ivTD: *in vitro* true disappearance (g/kg DM); dNDF: *in vitro* fiber digestibility (g/kg DM); ME: metabolisable energy (MJ/kg DM); TVFA: total volatile fatty acids (mmol); PF: partitioning factor (mg DM truly degraded/mL gas produced in 24 h); EMBP: efficiency of microbial biomass production (mg microbial mass/mL produced gas after 24 h); MBP: microbial biomass production (mg/g incubated feed) and ammonia concentration (mmol/L).

tended to be higher ($p = 0.089$) than that in the control silage, but addition of PEG had no effect on water-soluble carbohydrates content.

The GP parameters of the different silages are shown in Table 3. Using PEG during ensiling increased the asymptotic GP value ($p = 0.025$) and the GP rate ($p = 0.036$). The ME and TVFA contents were quadratically increased by PEG addition ($p = 0.022$ and $p = 0.024$, respectively), and ivTD and dNDF in PEG-treated silages tended to be higher than in the control ($p = 0.081$ and $p = 0.069$, respectively). Inclusion of PEG increased ammonia concentrations (L, $p < 0.001$; Q, $p < 0.003$). The GP_{24} was increased quadratically ($p = 0.024$) by increasing the PEG inclusion level. On the other hand, the PF and EMBP values were decreased quadratically ($p = 0.020$ and $p = 0.032$, respectively) by PEG inclusion. However, compared to the control silage, addition of PEG lowered MBP ($p = 0.035$).

The reduction observed in CP, ash, NDF or ADF content was due to the dilution effect of the added PEG to PP. The higher CP level and lower ammonia concentration in the control group could be explained by the lower CP degradation in the presence of tannins.

The higher lactic acid concentrations, as well as the lower pH values in PEG-treated silages, may be due to the inactivation of tannins by PEG, through the formation of tannin-PEG complexes. In addition, PEG treatment has been found capable of reversing the already formed tannin-substrates complexes, thus making substrates available for fermentation (Mangan, 1988). Salawu *et al*. (1999) reported that lactic acid concentration in perennial ryegrass silages treated with 0.5 and 5.0% of condensed tannins (on a DM basis) was reduced in comparison with the control. It is noteworthy that tannins may impair the growth of microorganisms responsible for silage fermentation. Rozes & Peres (1998) observed that the growth of *Lactobacillus plantarum* (a bacterium producing lactate during ensilage) was inhibited by high concentrations of tannins (1 g/L).

The GP in silages treated with PEG was higher than that of the control silage. Increases in GP due to the inclusion of PEG to tanniferous feeds have been previously reported by Baba *et al*. (2002). Also, inactivation of tannins through PEG binding enhances availability of nutrients resulting in increased microbial activity and GP (Makkar, 2005). The higher ME, TVFA and ammonia values in the silages treated with PEG was in agreement with the findings of Alipour & Rouzbehan (2007), who reported that addition of PEG (750 mg per 375 mg substrate in gas syringes) in grape pomace silage resulted in a higher ME, organic matter digestibility and ammonia concentrations than in the control silage. It should be taken into account that the extent of the limited ability of PEG to completely inhibit the negative effects of tannins on *in vitro* ruminal fermentation seems to depend on both the type of tannin and the microbial species in the rumen inoculum donor (Frutos *et al.,* 2004).

Baba *et al*. (2002) and Alipour & Rouzbehan (2007) reported that PF values of tanniniferous feeds were above the theoretical range of 2.75–4.41 for feedstuffs (Blümmel *et al*., 1997). However in the present study, the PF values of all treatments were within the theoretical range (3.85–4.33). This could be due to the fact that PEG decreased the PF value in the PP silages. Similarly, Alipour & Rouzbehan (2007) and Baba *et al*. (2002) reported decreased PF value due to PEG addition. Decreased of EMBP and MBP due to the inclusion of PEG were observed. These findings were similar to those of Bento *et al*. (2005) who also argued that EMBP was lower owing to the addition of PEG in comparison with without PEG addition. It should be noted that addition of PEG can affect the presence of some species of bacteria (Belenguer *et al.,* 2011) and therefore the rumen fermentation characteristics. It seems that the inclusion of PEG directed the nutrients toward the production of volatile fatty acids rather than to microbial biomass. Makkar *et al.* (1998) demonstrated that treating tannin-containing feeds with PEG increased the incorporation of ^{15}N into microbial protein but reduced EMBP.

Ensiling was found a suitable method for storing and using PP as animal feed in order to reduce the cost of diet and to prevent environmental pollution. Although the ME content of PP is low, it can be used to feed animals near to maintenance ME level, especially for sheep during feed scarcity. Further research is needed to investigate the effect of ensiled PP on productive performance of ruminants, such as milk or meat production, and also on their product quality.

References

Alipour D, Rouzbehan Y, 2007. Effects of ensiling grape pomace and addition of polyethylene glycol on in vitro gas production and microbial biomass yield. Anim Feed Sci Technol 137(1): 138-149. http://dx.doi.org/10.1016/j.anifeedsci.2006.09.020

AOAC, 1990. Official methods of analysis, vol. II, 15th ed. Association of Official Analytical Chemists, Arlington, VA, USA.

Baba ASH, Castro FB, Ørskov ER, 2002. Partitioning of energy and degradability of browse plants in vitro and the implications of blocking the effects of tannin by the addition of polyethylene glycol. Anim Feed Sci Technol 95: 93-104. http://dx.doi.org/10.1016/S0377-8401(01)00283-8

Barnett AG, Reid RL, 1957. Studies on production of volatile fatty acid production from fresh grass. J Agric Sci Camb 48: 315. http://dx.doi.org/10.1017/S0021859600031671

Belenguer A, Hervás G, Toral PG, Fondevila M, Frutos P, 2011. Is polyethylene glycol innocuous to the rumen bacterial community? A preliminary in vitro study. Anim Prod Sci 51: 990-995.http://dx.doi.org/10.1071/AN11041

Bento MHL, Makkar HPS, Acamovic T, 2005. Effect of mimosa tannin and pectin on microbial protein synthesis and gas production during in vitro fermentation of 15N-labelled maize shoots. Anim Feed Sci Technol 123/124: 365-377. http://dx.doi.org/10.1016/j.anifeedsci.2005.04.022

Blümmel M, 2000. Predicting the partitioning of fermentation products by combined in vitro gas volume-substrate degradability measurements: opportunities and limitations. In: Gas production: fermentation kinetics for feed evaluation and to assess microbial activity. Brit Soc Anim Sci, Penicuik, Midlothian, pp: 48-58.

Blümmel M, Makkar HPS, Becker K, 1997. In vitro gas production: a technique revisited. J Anim Physiol Anim Nutr 77: 24-34. http://dx.doi.org/10.1111/j.1439-0396.1997.tb00734.x

Broderick GA, Kang JH, 1980. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. J Dairy Sci 63: 64-75. http://dx.doi.org/10.3168/jds.S0022-0302(80)82888-8

Faithfull NT, 2002. Methods in agricultural chemical analysis: a practical handbook. CABI Publ, UK, 266 pp.http://dx.doi.org/10.1079/9780851996080.0000

France J, Dijkstra J, Dhanoa MS, Lopez S, Bannink A, 2000. Estimating the extent of degradation of ruminant feeds from a description of their gas production profiles observed in vitro: derivation of models and other mathematical considerations. Br J Nutr 83:143-150. http://dx.doi.org/10.1017/S0007114500000180

Frutos P, Hervas G, Giráldez F J, Mantecón AR, 2004. An in vitro study on the ability of polyethylene glycol to inhibit the effect of quebracho tannins and tannic acid on rumen fermentation in sheep, goats, cows and deer. Aust J Agric Res 55(11): 1125-1132. http://dx.doi.org/10.1071/AR04058

Makkar HPS (Ed.), 2000. Quantification of tannins in tree foliage. A laboratory manual for the FAO/IAEA co-ordinated research project on use of nuclear and related techniques to develop simple tannin assays for predicting and improving the safety and efficiency of feeding ruminants on tanniniferous tree foliage. Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Animal Production and Health Sub-Programme, FAO/IAEA Working Document, IAEA, Vienna, Austria.

Makkar HPS, 2003. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. Small Rumin Res 49: 41-256.http://dx.doi.org/10.1016/S0921-4488(03)00142-1

Makkar HPS, 2005. In vitro gas methods for evaluation of feeds containing phytochemicals. Anim Feed Sci Technol 123/124: 291-302. http://dx.doi.org/10.1016/j.anifeedsci.2005.06.003

Makkar HPS, Blümmel M, Becker, K, 1998. Application of an in vitro gas method to understand the effects of natural plant products on availability and partitioning of nutrients. Br Soc Anim Sci 22:147-150.

Mangan JL, 1988. Nutritional effects of tannins in animal feeds. Nutr Res Rev 1: 209-231. http://dx.doi.org/10.1079/NRR19880015

Markham R, 1942. A steam distillation apparatus suitable for micro-Kjeldahl analysis. Biochem J 36:790.

Menke KH, Steingass H, 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Anim Res Dev 28: 7-55.

Menke KH, Raab L, Salewski A, Steingass H, Fritz D, Schneider W, 1979. The estimation of the digestibility and metabolisable energy content of ruminant feeding stuffs from the gas production when they are incubated with rumen liquor in vitro. J Agric Food Sci 93: 217-222.

Moharrery A, 2007. The determination of buffering capacity of some ruminant's feedstuffs and their cumulative effects on TMR ration. Am J Anim Vet Sci 2(4): 72-78. http://dx.doi.org/10.3844/ajavsp.2007.72.78

Negro C, Tommasi L, Miceli A, 2003. Phenolic compounds and antioxidant activity from red grape marc extracts. Biores Technol 87(1): 41-44. http://dx.doi.org/10.1016/S0960-8524(02)00202-X

NRC, 2001. Nutrient requirements of dairy cattle, 7th rev ed. Nat Acad Press, Washington, D.C., USA.

Rozes N, Peres C, 1998. Effects of phenolic compounds on the growth and the fatty acid composition of Lactobacillus plantarum. Appl Microbiol Biotech 49(1): 108-111. http://dx.doi.org/10.1007/s002530051145

Salawu MB, Acamovic T, Stewart CS, Hvelplund T, Weisbjerg MR, 1999. The use of tannins as silage additive: effect on silage composition and mobile bag disappearance of dry matter and protein. Anim Feed Sci Technol 82: 243-259. http://dx.doi.org/10.1016/S0377-8401(99)00105-4

SAS, 2004.User's guide, V. 9.1: Statistics. SAS Institute, Cary, NC, USA.

Shabtay A, Eitam H, Tadmor Y, Orlov A, Meir A, Weinberg P, Weinberg ZG, Chen Y, Brosh A, Izhaki I, Kerem Z, 2008. Nutritive and antioxidative potential of fresh and stored pomegranate industrial byproduct as a novel beef cattle feed. J Agric Food Chem 56: 10063-10070. http://dx.doi.org/10.1021/jf8016095

Taghavi-Nezhad M, Alipour D,Torabi Goudarzi M, Zamani P, Khodakaramian G, 2011. Dose response to carvone rich essential oils of spearmint (Mentha spicata L.): in vitro ruminal fermentation kinetics and digestibility. J Agr Sci Tech 13: 1013-1020.

Taylor KA, 1996. A simple colorimetric assay for muramic acid and lactic acid. Appl Biochem Biotechnol 561: 49-58. http://dx.doi.org/10.1007/BF02787869

Van Soest, PJ, Robertson, JB, Lewis BA, 1991. Methods for dietary fiber, neutral detergent fiber and non-starch carbohydrates in relation to animal nutrition. J Dairy Sci 74: 3583-3597. http://dx.doi.org/10.3168/jds.S0022-0302(91)78551-2