Water-Soluble Reaction Products from Ozonolysis of Grasses

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Ozone has been used to pretreat agricultural byproducts with the aim of increasing nutritive value for ruminants. However, not all treatments with ozone result in enhanced digestibility, suggesting reaction products from ozone treatment of plants might inhibit rumen microbial activity. Coastal Bermuda grass (*Cynodon dactylon* L. Pers.) (CBG) and Kentucky-31 tall fescue (*Festuca arundinacea* Schreb.) (K-31) were treated with ozone and the water-soluble products determined. The following acids were identified: caproic, levulinic, *p*-hydroxybenzoic, vanillic, azelaic, and malonic. In addition, vanillin and *p*-hydroxybenzaldehyde were also identified. Ozone treatment of the cell walls of CBG produced mainly *p*-hydroxybenzoic acid, vanillic acid, azelaic acid, *p*-hydroxybenzaldehyde, and vanillin. Ozone treatment of K-31 cell walls produced levulinic acid in addition to those products found from CBG cell walls. The production of vanillin and *p*-hydroxybenzaldehyde, which have been shown to be especially toxic to rumen microorganisms, offers an explanation for the negative affects of ozone treatment on forage.

Ozone is a highly reactive molecule whose chemistry and applications have been extensively reviewed (Bailey, 1978). Ozone has been used to pretreat a number of agricultural byproducts with the aim of improving their nutritive value for ruminants. Neely (1984) has evaluated a number of factors affecting ozone pretreatment of agricultural byproducts including ozone flow rate, concentration, exposure of substrate to the gas, particle size, and moisture content of the substrate. Ozonolysis improved the digestibility of wheat straw (*Triticum aestivum* L.) (Ben-Ghedalia and Miron, 1981), cotton straw (*Gossypium hirsutum* L.) (Ben-Ghedalia et al., 1983), grape branches (Ben-Ghedalia, 1982), and mesquite (*Proscopisis sp.*) (Bryant et al., 1984). Ben-Ghedalia and Shefet (1983) have shown that ozone treatment makes cell wall polysaccharides more available for digestion by rumen microorganisms when compared to untreated or sodium hydroxide treated straw in feeding trials using cannulated sheep.

In earlier work, we reported (Akin and Morrison, 1988) that ozone treatment improved the digestibility of Coastal Bermuda grass and Kentucky-31 tall fescue. In accompanying microscopic studies to define the effects on specific plant cell walls of leaves and stems, we found that ozone treatment increased the availability of sclerenchyma ring and xylem tissue, which are the most resistant tissues to biodegradation. Despite the increased digestibility of ozone-treated plants, some tissues showed no biodegradation and no adherence of rumen bacteria, suggesting a localized toxicity to rumen microorganisms. From this study, we concluded that ozone reacted with the most highly lignified plant cell walls and the potential existed to produce as byproducts low molecular weight phenolic compounds inhibitory to rumen microorganisms.

Despite improvements in quality observed in some studies, other researchers have reported potential problems with ozone-treated feeds. Shefet and Ben-Ghedalia (1982) found that ozone treatment of feeds resulted in a 12–24-h lag in the digestion of plant cell wall polysaccharides. Similarly, other researchers reported that ozonized triticale was not degraded by rumen microorganisms until the byproducts of ozonation were washed away (Narasimhalu et al., 1989). The possibility existed that ozonation produced toxic phenolic monomers that limited plant digestibility by rumen microorganisms.

The toxic effects of low molecular weight phenolic compounds on rumen microorganisms in vitro are established. Phenolic acids and aldehydes inhibit the growth and plant cell wall degrading activity of pure and mixed populations of bacteria (Borneman et al., 1986; Jung, 1985; Jung et al., 1983a,b; Theodorou et al., 1987), activity of plant cell wall degrading enzymes of rumen bacteria (Martin and Akin, 1988), growth and degradative activity of anaerobic fungi (Akin and Rigsby, 1987), and viability of rumen protozoa (Akin, 1986). With the substantial affect of phenolic compounds on rumen microorganisms, it is highly likely that the lag in degradation of ozonized forage results from a toxic affect on fiber-degrading microbes. However, to date no research has been reported that establish types of phenolic products produced in feeds having undergone ozone pretreatment.

As a part of our ongoing research on the nutritive value of plants, ozone has been used to pretreat forage grasses with the aim of assessing its use for improving digestibility in the ruminant. The present work reports on the nature of products produced by the reaction of ozone with Coastal Bermuda grass and Kentucky-31 tall fescue.

MATERIALS AND METHODS

Plant Samples. Field-grown Coastal Bermuda grass [*Cynodon dactylon* (L.) Pers.] (CBG) shoots were 6-week-old regrowth harvested from plots near Athens, GA. Kentucky-31 tall fescue (*Festuca arundinacea* Schreb.) (K-31) shoots were 4–6-week-old regrowth from fields near Lexington, KY (Akin and Burdick, 1975). All plants were frozen quickly after harvesting and stored at −10 °C. All samples were treated in duplicate, and statistical significance was determined by *t*-test (Steel and Torrie, 1960).

Ozonolysis of Whole Forage Material. Samples were prepared from freeze-dried material that had been ground to pass a 1-mm (20-mesh) screen. The ground sample was adjusted to about 50% moisture by the addition of an equal weight of water.

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Table I. Water-Soluble Products from Untreated and Ozone-Treated Coastal Bermuda Grass and Kentucky-31 Tall Fescue (mg/100 g DW)[a]

	coastal Bermuda grass					Kentucky-31 tall fescue				
		ozone treated					ozone treated			
	untreated whole grass	whole grass	water-extracted residue	filtrate[b]	cell walls	untreated whole grass	whole grass	water-extracted residue	filtrate[b]	cell walls
caproic acid		22 ± 7	25 ± 7				35 ± 5	29 ± 5		
levulinic acid		15 ± 12**	10 ± 1***			3 ± 1	84 ± 8**	123 ± 12***		65 ± 30
p-hydroxybenzoic acid	5 ± 1	37 ± 1**	103 ± 16**		84 ± 10	5 ± 1	14 ± 7**	36 ± 7**		92 ± 7
p-hydroxybenzaldehyde	2 ± 2	50 ± 10**	69 ± 1***		139 ± 23	13 ± 1	9 ± 2**	13 ± 1***		154 ± 84
vanillin		7 ± 0	14 ± 1		36 ± 20	3 ± 2				45 ± 31
vanillic acid	3 ± 4	5 ± 0	9 ± 1		10 ± 1	5 ± 1		9 ± 9		21 ± 8
azelaic acid	9 ± 1	127 ± 78	144 ± 1*	28 ± 3	14 ± 1	11 ± 1	224 ± 29	160 ± 7*	29 ± 5	30 ± 9
p-coumaric acid	21 ± 1					43 ± 12				
ferulic acid	9 ± 5					6 ± 1				
palmetic acid	5 ± 1					16 ± 8			9 ± 3	
unknown		16 ± 2	19 ± 2	12 ± 16			31 ± 9	19 ± 7	56 ± 21	

[a] Mean of duplicate treatments. Levels of significance: *, $P < 0.1$; **, $P < 0.05$; ***, $P < 0.01$. Statistical comparisons made on like treatments between grasses. [b] Based on dry weight of grass before extraction.

Samples were sealed and allowed to equilibrate overnight at 1 °C and then stored at −20 °C until used. Dry matter (DM) analyses were determined by drying moistened and ground samples in a vacuum oven at 60 °C for 6 h. Ozonolysis was carried out on a Welsbach Model T-408 ozone generator (Welsbach Ozone Systems, Sunnyvale, CA) as described earlier (Akin and Morrison, 1988). Briefly, ozonolysis of whole material was conducted on 5 g of sample in a 500-mL filter flask fitted with a plug of glass wool large enough to cover the vacuum nipple. A rubber stopper fitted with a glass tube was placed in the top of the flask. The flask was inverted and ozone introduced through the glass tube for the times specified (60 min for whole forage material). There was no attempt to maximize reaction conditions.

On completion of ozonolysis, the samples were stirred with distilled water (150 mL) for 6 h. After filtration through Schleicher & Schuell 588 (24-cm) prepleated filter paper, the residue was washed with an additional 20 mL of distilled water. The filtrate (pH 3.5) was adjusted to pH 2.5 with 2 M HCl. Syringaldehyde (1–2 mg) was added as an internal standard, and the mixture was extracted with freshly redistilled diethyl ether (2 × 50 mL). Before the second extraction, saturated NaCl solution (20 mL) was added. The combined ether layers were washed with saturated NaCl solution (20 mL), and the ether was evaporated under vacuum. Acetone was added to azeotrope the last traces of water. The residue was dissolved in ether (1 mL) and transferred to a vial and the solvent evaporated under a stream of nitrogen. The residue was dissolved in dry pyridine (50 μL) and silylated with bis(trimethylsilyl)trifluoroacetamide (BSTFA) (50 μL).

Analysis of Whole, Untreated Forage. Phenolic acids in the untreated forage and cell walls were isolated by published methods (Hartley et al., 1988) and analyzed by gas chromatography with sinapic acid added as an internal standard. The compositions of the water-soluble extracts of the untreated forages were determined by preparing untreated samples using the same workup described above for the water-soluble products of ozone-treated materials. Fatty acid compositions of the untreated forages were determined by gas chromatographic measurements of the fatty acid methyl esters (Chapman et al., 1976).

Ozonolysis of Plant Extract and Residue. Distilled water (150 mL) was added to 4 g of whole, ground CBG or K-31 and the mixture stirred for 6 h. The mixture was filtered as above. The filtrates were ozonized at 0 °C for 15 min, adjusted to pH 2.5, and worked up as described above. The residues from the water extract were dried under vacuum, rehydrated to 50% moisture, treated with ozone for 30 min at room temperature, and worked up under the same conditions described above for the whole plant samples.

Cell Wall Preparation and Ozonolysis. Cell wall material was prepared by boiling 1 g of dried, whole, ground forage with neutral detergent solution (100 mL) (Van Soest and Wine, 1967) without the addition of sodium sulfite and Decalin (Hartley et al., 1974) for 1 h. The mixture was filtered through a coarse sinter, washed with distilled water (750 mL) followed by acetone until no color remained in the filtrate, and then washed with diethyl ether (100 mL). The residue (cell walls) was dried overnight at 30 °C in a vacuum oven. Cell walls (about 0.6 g dry weight) were adjusted to 50% moisture, ozonized for 15 min, and worked up as described above.

Gas Chromatographic Analysis. Gas chromatographic (GC) analysis was conducted on a Perkin-Elmer Sigma 2100 gas chromatograph with an Analabs GB-1 (50 m × 0.25 mm (i.d.)) fused silica column (0.25-μm film) and a flame ionization detector (250 °C) with helium as the carrier gas. The injector temperature was 250 °C. The column oven was set at an initial temperature of 100 °C and programmed to 220 °C at 5 °C/min. *p*-Coumaric and ferulic acids were determined with the column temperature at 220 °C, isothermal. Data were collected and recorded on a Perkin-Elmer LCI-100 laboratory computing integrator.

GC–Mass Spectral Analysis. An Extrel Model C50/400 (Pittsburgh, PA) quadrapole mass spectrometer interfaced with a Perkin-Elmer Sigma 300 gas chromatograph (Norwalk, CN) equipped with a cold on-column injector was used. Chromatographic separations were made on a 20 m × 0.32 mm (o.d.) fused silica capillary column coated with General Electric SE-54 (0.10 μm). Helium was used as a carrier gas at 0.5 psi. The column oven was programmed from 80 to 220 °C at 7 °C/min and held for 40 min. Mass spectrometer conditions: ion source temperature, 150 °C; scan rate, 200 aMU/s; ionizing voltage, 70 eV. Data were collected with a Technivent software and interface (St. Louis, MO) and processed on an IBM PC (640K, 40-Mbyte disk). Compounds were considered to be positively identified when their mass spectra and GC retention times agreed with those of authentic samples that were obtained from commercial sources.

RESULTS AND DISCUSSION

Table I shows the amounts of compounds extracted from unozonized whole plants and from ozonized fractions of CBG and K-31. Ozonation produced *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid in all samples, except the water-soluble extracts, and significantly higher concentrations of these compounds were present in the products from CBG than in K-31. These two compounds accounted for a total of 87 and 172 mg/100 g dry weight (DW) for grass and water-extracted residue, respectively, for CBG as compared to 23 and 49 mg/100 g DW for K-31. However, these compounds were produced in nearly equal concentrations from the cell wall material (223 and 246 mg/100 g DW for CBG and K-31, respectively).

The same trends were seen for vanillin and vanillic

Table II. Phenolic and Fatty Acids in Unozonized Coastal Bermuda Grass and Kentucky-31 Tall Fescue (mg/100 g DW)[a]

	coastal Bermuda grass		Kentucky-31 tall fescue	
acid	whole grass	cell walls	whole grass	cell walls
palmetic	23 ± 19		164 ± 12	
linoleic	87 ± 5		106 ± 17	
linolenic	205 ± 2		259 ± 6	
p-coumaric	2043 ± 57	1697 ± 183	710 ± 127	919 ± 20
ferulic	835 ± 101	1081 ± 14	608 ± 16	904 ± 63

[a] Means of duplicate analyses.

acid. However, concentrations tended to be higher in cell walls from K-31. The total concentrations of vanillin and vanillic acid were much lower than for *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid in each grass.

Caproic and azelaic acids were also identified in ozonized plants. Azelaic acid was obtained from all treatments and was present in higher concentrations in K-31. Caproic acid was not present in unozonized plants and was present generally only in the grass and water-extracted residues, occurring in equal concentrations for CBG and K-31. Malonic acid was also present in trace amounts in all fractions except the cell walls (not shown).

Levulinic acid was identified in grass and water-extracted fractions of both CBG and K-31 as well as in the cell walls of K-31 after ozonation, but was not found in unozonized CBG. Significantly higher concentrations were found in the K-31 samples for all fractions.

The aromatic aldehydes and acids are probably the result of ozone oxidation of *p*-coumaric and ferulic acids, the principal phenolic acids in these grasses (Table II). Kolsaker and Bailey (1967) investigated the ozonation of substituted cinnamic acids and esters. The expected aldehydes and acids from cleavage of the double bond were produced as well as unexpected phenols resulting from an aryl migration and rearrangement of the intermediate zwitterion. In our study, hydroquinone was present in trace amounts in the products from both grasses. This compound could arise from oxidation and rearrangement of ester-linked *p*-coumaric acid. No methoxyhydroquinone, which could be produced from ester-linked ferulic acid, was found. This observation agrees with the migratory aptitude of the substituted aryl groups reported by Kolsaker and Bailey (1967).

In this study, *p*-coumaric and ferulic acids as determined by treatment of whole grass and cell walls with 1 M NaOH were present in total concentrations of 1318 and 2878 mg/100 g of whole K-31 and CBG, respectively (Table II). In cell walls, their total concentrations were 1823 and 2778 mg/100 g for K-31 and CBG, respectively, with the *p*-coumaric acid concentration in CBG being almost twice that in K-31. The higher amounts of *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, vanillin, and vanillic acid suspected to arise from these acids in CBG agree with the higher percentage of *p*-coumaric and ferulic acids in CBG. However, it appears that these acids are more available for oxidation by ozone in whole CBG than in K-31. In the cell walls alone, the concentration of oxidation products (Table I) is only slightly but not significantly higher for K-31 compared with CBG, suggesting a more equal availability for oxidation in the isolated cell walls of the two plants.

Ozonolysis of cinnamic acid or its esters produced oxalic acid and the monoesters, respectively. Acid hydrolysis of the monoesters also produced oxalic acid (Kolsaker and Bailey, 1967). The conditions reported by these investigators for acid hydrolysis were much more severe than those used in the present study. Although it would be expected that the monoester would be produced if the phenolic acids are ester-linked, no oxalic acid was found, probably as a result of the mild conditions used.

Caproic and azelaic acids are probably produced by the oxidation of linoleic and linolenic acids (Table II). Chapman et al. (1976) have shown that the total fatty acid content and their individual percentages varied in CBG from 0.35 to 2.4% DM with the percentage of linolenic ranging from 34 to 58.8%. Linoleic acid remained fairly constant at 12–24% of the total fatty acids. The variation was dependant on crop maturity and diurnal affects. In our study, fatty acids accounted for about 0.50% of the dry weight for both CBG and K-31. The linoleic acid content was 87 mg/100 g DW for CBG and 106 mg/100 g DW for K-31. Linolenic acid was present at a concentration of 205 mg/100 g DW for CBG and 259 mg/100 g DW for K-31 (Table II).

Caproic acid results from the oxidation of the C-12 double bond in linoleic acid (Privett and Nickell, 1966). Azelaic acid could result from the oxidation of the C-9 double bond in either linoleic or linolenic acids (Benton et al., 1959). Malonic acid could be produced from the oxidation of the C-9 and C-12 or the C-12 and C-15 double bonds. The presence of malonic acid further suggests that linolenic and linoleic acids are the source of the aliphatic carboxylic acids. These two fatty acids are probably not associated with the cell walls and appear to be more available for oxidation in K-31 than in CBG. Azelaic acid occurs naturally in ryegrass (Shelvey and Koziol, 1986) and was present as the free acid in trace amounts in both CBG and K-31. Ozonolysis of cell walls did produce azelaic acid, but the origin of the acid has not yet been determined.

Levulinic acid is known to be produced by acid hydrolysis of hexoses (Leonard, 1956; Ploetz, 1941). However, the reaction conditions needed to cause this conversion are more severe than those used in our study.

The compound indicated as unknown in Table I elutes between vanillin and *p*-hydroxybenzoic acid on the gas chromatograph and gave the following mass spectrum [major ions, m/z (rel intens)]: 75 (100), 73 (93), 117 (37), 55 (20), 129 (19), 74 (14), 67 (12), 95 (9), 131 (8), 76 (7), 27 (7), 77 (7), 211 (4), 229 (1). It is one of the main products found from ozonolysis of the water-soluble fraction.

The toxic effects of various phenolic compounds to rumen microorganisms have been well established in in vitro studies by several researchers (Borneman et al., 1988; Jung, 1985; Jung et al., 1983a,b; Theodorou et al., 1987). Studies have concentrated on phenolic acids, and in particular *p*-coumaric and ferulic acids since these are the major saponifiable phenolic compounds in grasses (Table II; Akin, 1986). However, two studies indicated that phenolic aldehydes (i.e., vanillin and *p*-hydroxybenzaldehyde) were more toxic than the acids (Borneman et al., 1986; Jung, 1985). These data, as well as those presented in this study on the products of ozonolysis, help clarify digestibility studies, where either a lag or total prevention in the digestibility of cell wall monosaccharides occurred (Shefet and Ben-Ghedalia, 1982) and a washing away of the products of ozonolysis restored digestibility (Narasimhula et al., 1989). The fact that cell wall monosaccharides were digested at a greater rate after this lag is explained from pyrolysis mass spectrometry studies (Morrison et al., 1990) of the residues remaining after water-washing of ozone-treated CBG and K-31 cell walls. The results suggest that this residue is almost pure polysaccharide with little phenolic material remaining. With the

phenolic matrix removed, the cell wall sugars would be much more available for digestion. These studies strongly suggest that phenolic aldehydes resulting from oxidation of phenolic compounds such as *p*-coumaric and ferulic acids contribute to the inhibition in digestibility resulting from ozonolysis of feeds.

Previous studies showing the toxic nature of phenolic aldehydes evaluated concentrations higher than those found in this study (Borneman et al., 1986; Jung, 1985). However, data on the concentrations in the microenvironment of the microbe/cell wall during degradation are not available. Therefore, at present the effect of phenolic compounds at the cell wall level cannot be determined. Likely, various concentrations of phenolic compounds with potential toxicity to rumen microbes will depend upon the amount and availability to oxidation of phenolic compounds within cell wall types (Akin, 1986) and upon the degree of ozonation (Narashimhula et al., 1989).

The effects of malonic, azelaic, and levulinic acids on rumen microorganisms are not known. Fatty acids (i.e., oleic, lauric, and caproic) have been reported to influence the growth of rumen bacteria (Henderson, 1973) and to reduce the volatile fatty acid production and alter the proportions of products (Chalupa et al., 1984). With the information to date, the potential toxic effect of ozonation products on fiber degradation can be linked to phenolic aldehydes while the effects of the other compounds are yet to be determined.

LITERATURE CITED

Akin, D. E. Interaction of Ruminal Bacteria and Fungi with Southern Forages. *J. Anim. Sci.* **1986**, *63*, 962–977.

Akin, D. E.; Burdick, D. Percentage Tissue Types in Tropical and Temperate Grass Leaf Blades and Degradation of Tissues by Rumen Microorganisms. *Crop. Sci.* **1975**, *15*, 661–668.

Akin, D. E.; Rigsby, L. L. Mixed Fungal Populations and Lignocellulosic Tissue Degradation in the Bovine Rumen. *Appl. Environ. Microbiol.* **1987**, *53*, 1987–1995.

Akin, D. E.; Morrison, W. H., III. Ozone Treatment of Forage: Structure and Digestibility of Different Lignified Cell Walls. *Crop Sci.* **1988**, *28* (2), 337–342.

Bailey, P. S. *Ozonation In Organic Chemistry*; Academic Press: New York, 1978; Vol. 1.

Ben-Ghedalia, D. Degradation of Cell-Wall Monosaccharides of Chemically Treated Grape Branches by Rumen Microorganisms. *Eur. J. Appl. Microbiol. Biotechnol.* **1982**, *16*, 142–145.

Ben-Ghedalia, D.; Miron, J. J. Effects of Sodium Hydroxide, Ozone, and Sulfur Dioxide on the Composition and *In-Vitro* Digestibility of Wheat Straw. *Sci. Food Agric.* **1981**, *32*, 224–228.

Ben-Ghedalia, D.; Shefet, G. J. Chemical Treatment for Increasing the Digestibility of Cotton Straw. II. *J. Agric. Sci.* **1983**, *100*, 401–406.

Ben-Ghedalia, D.; Shefet, G. J.; Dror, Y. Chemical Treatment for Increasing the Digestibility of Cotton Straw. I. *J. Agric. Sci.* **1983**, *100*, 393–400.

Benton, F. L.; Kiess, A. A.; Narwood, H. J. Ozonolysis as a Method for Establishing the Position of Olefinic Linkage. *J. Am. Oil Chem. Soc.* **1959**, *36*, 457–460.

Borneman, W. S.; Akin, D. E.; Van Eseltine, W. P. Effects of Phenolic Monomers on Rumen Bacteria. *Appl. Environ. Microbiol.* **1986**, *52*, 1331–1339.

Bryant, E. C.; Mills, T.; Pitts, J. S.; Carrigan, M.; Wiggers, E. P. Ozone Treated Mesquite for Supplementing Steers in West Texas. *J. Range Manag.* **1984**, *37*, 420–422.

Chalupa, W.; Richabaugh, B.; Kornfeld, D. S.; Sklan, D. L. Rumen Fermentation *In Vitro* as Influenced by Long Chain Fatty Acids. *J. Dairy Sci.* **1984**, *67*, 1439–1444.

Chapman, G. W.; Robertson, J. A.; Burdick, D. Fatty Acid and Chlorophyll Levels in Coastal Bermudagrass During the Day and During Maturation. *Physiol. Plant* **1976**, *36*, 66–70.

Hartley, R. D.; Jones, E. C.; Fenlon, J. S. Prediction of the Digestibility of Forage by Treatment of Their Cell Walls with Celluloytic Enzymes. *J. Sci. Food Agric.* **1974**, *25*, 947–954.

Hartley, R. D.; Whatley, F. R.; Harris, P. J. 4,4′-Dihydroxytruxillic Acid as A Component of Cell Walls of Lolium Multiflorum. *Phytochemistry* **1988**, *27*, 349–351.

Henderson, C. J. The Effects of Fatty Acids on Pure Cultures of Rumen Bacteria. *J. Agric. Sci.* **1973**, *81*, 107–112.

Jung, H. G. Inhibition of Structural Carbohydrate Fermentation by Forage Phenolics. *J. Sci. Food Agric.* **1985**, *36*, 74–80.

Jung, H. G.; Fahey, G. C.; Garst, J. E. Simple Phenolic Monomers of Forage and Effects on *In Vitro* Fermentation on Cell Wall Phenolics. *J. Anim. Sci.* **1983a**, *57*, 1294–1305.

Jung, H. G.; Fahey, G. C.; Mercen, N. R. Effects of Ruminant Digestion and Metabolism on Phenolic Monomers of Forages. *Br. J. Nutr.* **1983b**, *50*, 637–651.

Kolsaker, A. T.; Bailey, P. S. Ozonation of Compounds of the Type ArCHCHG; Ozonation In Methanol. *Acta. Chem. Scand.* **1967**, *21*, 537–546.

Krumbolz, L. R.; Bryant, M. P. *Syntrophococcus Sucromutans* sp. nov. gen. nov. Uses Carbohydrates as Electron Doners and Formate, Methoxymonobenzenoids or *Methanobreibacter* as Electron Acceptor Systems. *Arch. Microbiol.* **1986**, *143*, 313–318.

Leonard, R. H. Levulinic Acid as a Basic Chemical Raw Material. *Ind. Eng. Chem.* **1956**, *48*, 1331–1341.

Martin, S. A.; Akin, D. E. Effects of Phenolic Monomers on the Growth and β-glucosidase of *Bacteriodes Ruminicola* and on the Carboxymethylcellulase, β-glucosidase, and xylanase from *Bacteriodes Succinogenes*. *Appl. Environ. Microbiol.* **1988**, *54*, 3019–3022.

Morrison, W. H., III; Scheijen, M. A.; Boon, J. J. Pyrolysis Mass Spectrometry of Coastal Bermudagrass (*Cynodon dactylon* (L.) Pers.) and Kentucky-31 Tall Fescue (*Festuca arundinacea* Schreb.) Cell Walls and Their Residues After Ozonolysis and Base Hydrolysis. *Anim. Feed Sci. Technol.* **1990**, in press.

Narasimhalu, P.; Graham, H.; Åman, P. Effects of Ozone Treatment on the Chemical Composition and *In-Vitro* Degradation of Triticale Internodes and Red Clover Stems. *Anim. Feed Sci. Technol.* **1989**, in press.

Neely, W. C. Factors Affecting the Pretreatment of Biomass With Gaseous Ozone. *Biotechnol. Bioeng.* **1984**, *26*, 59–65.

Ploetz, T. The Formation of Levulinic Acid From Carbohydrates. *Naturwessenschaften* **1941**, *29*, 707–708.

Privett, O. S.; Nichell, E. C. Recent Advances in the Determination of the Structure of Fatty Acids via Ozonolysis. *J. Am. Oil Chem. Soc.* **1966**, *43*, 393–400.

Shefet, G. J.; Ben-Ghedalia, D. Effects of Ozone and Sodium Hydroxide Treatments on the Degradability of Cotton Straw Monosaccharides by Rumen Microorganisms. *Eur. J. Appl. Microbiol. Biotechnol.* **1982**, *15*, 47–51.

Shelvey, J. D.; Koziol, M. J. Seasonal and SO_2-Induced Changes in Epicuticular Wax of Ryegrass. *Phytochemistry* **1986**, *25*, 415–420.

Steel, R. G. D.; Torrie, J. H. *Principles and Procedures of Statistics*; McGraw-Hill: New York, 1960.

Theodorou, M. K.; Gascoyne, J. D.; Akin, D. E.; Hartley, R. D. Effects of Phenolic Acids and Phenolics From Plant Cell Walls on Rumenlike Fermentation in Consecutive Batch Culture. *Appl. Environ. Microbiol.* **1987**, *53*, 1046–1050.

Van Soest, P. J.; Wine, R. H. Use of Detergents in the Analysis of Fiberous Feeds. IV Determination of Plant Cell Wall Constituents. *J. Assoc. Off. Agric. Chem.* **1967**, *50*, 50–55.

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