Otto, S. *Atti Simp. Chim. Anticrittogamici Piacenza* **1980**, *1*, 109.
Piglionica, V.; Ferrara, G. *Dif. Piante* **1979**, *4*, 207.
Ronco, C.; Barberis, E.; Sapetti, C. *Atti Simp. Chim. Antiparassitari Piacenza* **1981**, *2*, 98.
Selva, A.; Marchini, G. F.; Griffini, A.; Molinari, G. P. *Atti Simp. Chim. Antiparassitari Piacenza* **1981**, *2*, 139.
Siegel, M. R. *Phytopathology* **1975**, *65*, 219.
SISS (Società Italiana della Scienza del Suolo) "Metodi Normalizzati di analisi del suolo"; Tipografia G. Capponi: Firenze, Italy, 1976.
Wainwright, M. *Z. Pflanzenernaehr. Bodenkd.* **1977**, *140*, 587.
Zanini, E.; Barberis, E.; Ronco, C. *J. Agric. Food Chem.* **1980**, *28*, 464.
Zweig, G. "Analytical Methods for Pesticide, Plant Growth Regulators, and Food Additives"; Academic Press: New York, 1964; Vol. IV.

Received for review October 13, 1981. Accepted July 7, 1982.

A Series of Pyromellitic Diimides That Improve the Efficiency of Rumen Fermentation

Bruce O. Linn,* Lynn M. Paege, Patrick J. Doherty, Richard J. Bochis, Frank S. Waksmunski, Peter Kulsa, and Michael H. Fisher

A number of pyromellitic diimides were prepared and found to alter rumen fermentation in a potentially beneficial way. In vitro rumen test procedures and chemical syntheses for preparation of novel unsymmetrical N-substituted pyromellitic diimides were developed. Structure–activity investigations showed that a variety of N substituents could be tolerated while retaining high activity. The most potent compounds in the series had ID_{50} values for methane suppression of approximately 1 μg/mL and blocked methane production completely at higher levels. Volatile fatty acid composition was shifted from acetic to propionic and butyric acids. Two compounds, pyromellitic diimide and pyromellitic *N*-(2-hydroxyethyl)diimide, have been selected for further study in sheep and cattle.

The rumen, in ruminating animals, provides an ecosystem in which microorganisms, living at a pH between 5.5 and 7.0 in an anaerobic environment, can metabolize incoming materials into nutrients to be used by the host. The principal end products of carbohydrate metabolism in this system are volatile fatty acids (VFA's) such as acetic acid, propionic acid, and butyric acid, which are absorbed by the host and the gases carbon dioxide and methane. Methane is formed by the reduction of carbon dioxide by hydrogen. An outline of the principal pathways of carbohydrate metabolism is shown in Scheme I [adapted from Hungate (1966) and Leng (1970)]. Methane is expelled.

A typical rumen fermentation may be approximated by the equation (Wolin, 1974)

$$57.5C_6H_{12}O_6 \rightarrow 65CH_3COOH + 20CH_3CH_2COOH + 15CH_3CH_2CH_2COOH + 60CO_2 + 35CH_4 + 25H_2O \quad (1)$$

If one considers energy utilization and conservation via glycolysis, the following generalization applies (Hungate, 1966):

$$\underset{672\ \text{kcal/mol}}{C_6H_{12}O_6} \rightarrow \underset{420\ \text{kcal/mol}}{CH_3COOH} + \underset{734\ \text{kcal/mol}}{CH_3CH_2COOH} + \underset{524\ \text{kcal/mol}}{CH_3CH_2CH_2COOH} + CH_4 \quad (2)$$

Thus, the elevation of the levels of propionic and butyric acids, with concomitant reduction of acetic acid, should improve the energetics of rumen fermentation and hence enhance feed efficiency in ruminant animals (Hungate, 1966; Chalupa, 1977). Any reduction in the production of methane, a waste product, should result in energy conservation.

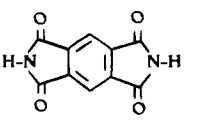
Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065.

There have been a number of publications describing the enhancement of feed efficiency in sheep and cattle by monensin (Davis and Erhart, 1976; Perry et al., 1976; Potter et al., 1976a,b; Raun et al., 1976). Part of this improvement was attributed to a favorable alteration of rumen energetics by elevation of propionic acid and reduction of acetic acid production (Richardson et al., 1976).

BIOLOGICAL EVALUATION

An approach taken in these laboratories was to search for compounds that would suppress methane production in rumen fluid while lowering acetic acid and raising propionic acid levels. Such compounds would be expected to improve feed efficiency in cattle and sheep. A rapid, high-capacity, in vitro, batch-type rumen fermentation system was developed, utilizing strained rumen fluid and a high-energy feed ration (see Experimental Section, Test Procedures). Selected compounds were tested at 250 μg/mL. Those compounds showing better than 70% methane inhibition, and a shift of acetic acid to propionic, butyric, or valeric acids were retested at lower levels. The most active compounds were titrated in an 18-h assay in order to determine the dose at which methane production was inhibited to 50% of control (ID_{50}) and the VFA composition at this level.

Pyromellitic diimide (1) was highly active in these procedures and a structure–activity investigation was undertaken.



I

CHEMISTRY

The synthesis of pyromellitic symmetrically N,N′-disubstituted diimides (II) from pyromellitic dianhydride

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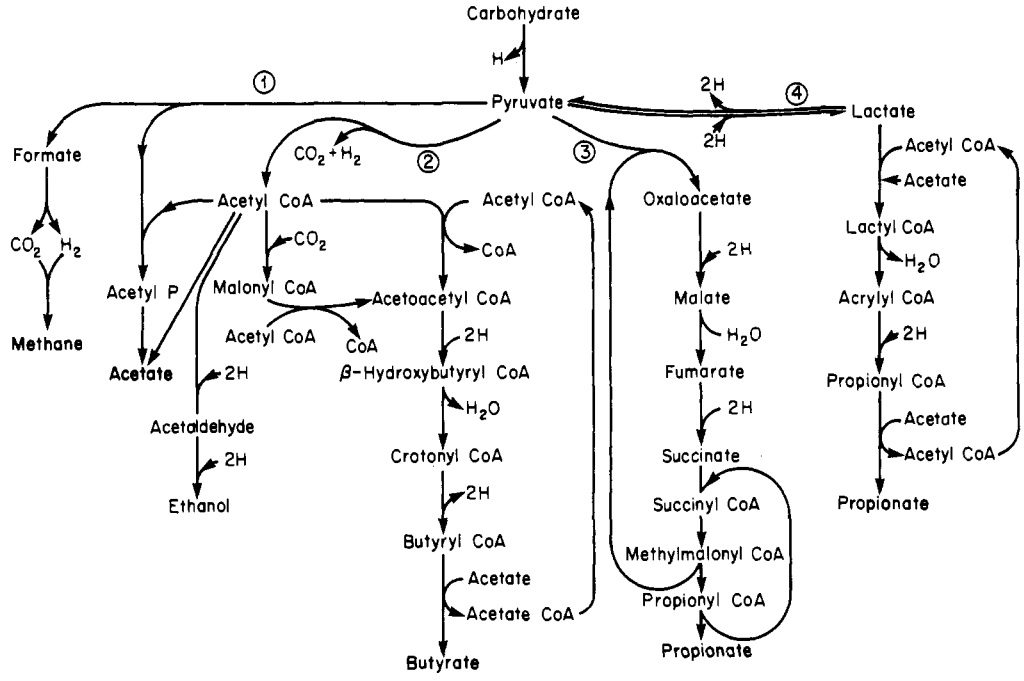
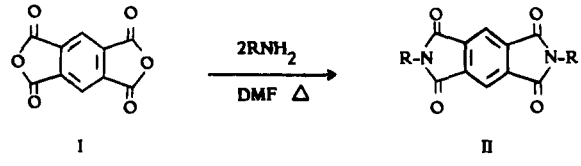
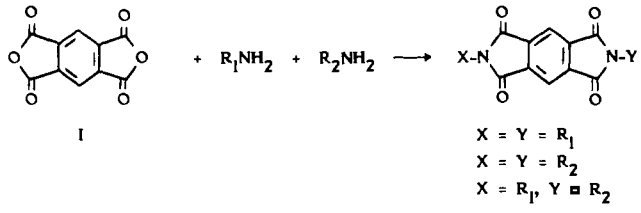
Scheme I



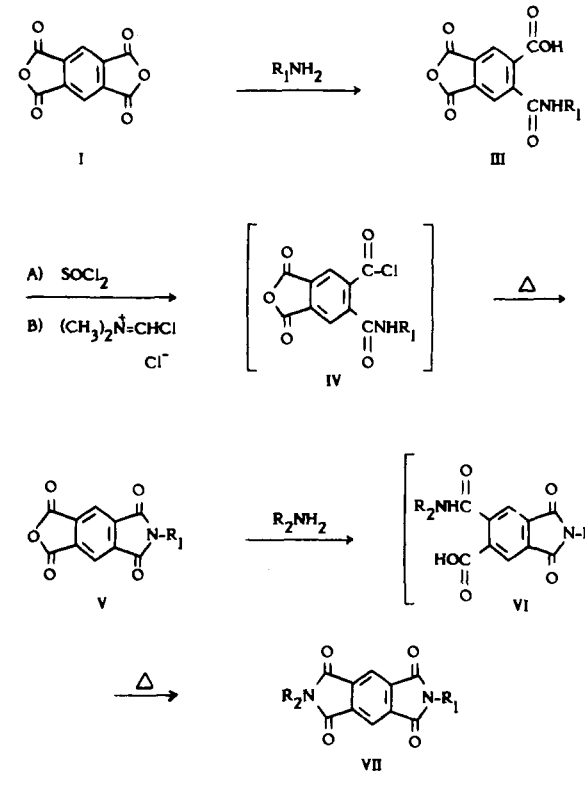
Scheme II



Scheme III



Scheme IV



and a variety of amines or amine generators has been reported (Meyer and Steiner, 1914; Gitis et al., 1966; Ibing and Neubold, 1972). Pyromellitic diimides of this type (Table II) described in this publication were obtained by heating a slight excess of the appropriate amine with pyromellitic dianhydride (I), in DMF, as shown in Scheme II.

Pyromellitic unsymmetrically N-substituted diimides (Tables III and IV) are more difficult to synthesize and few have been reported (Smith and Popoff, 1963). Smith and Popoff describe unsymmetrical pyromellitic N,N'-disubstituted diimides prepared in silicone fluids for use as lubricant stabilizers. Experimentally the products were not isolated and undoubtedly were obtained as mixtures that contained the other two possible symmetrically disubstituted products as shown in Scheme III.

The synthetic procedures developed in our studies provide the unsymmetrical N,N'-disubstituted pyromellitic diimides (VII, $R_1 \neq R_2$) as pure entities and also allow for the first time the preparation of the unsymmetrical N-monosubstituted pyromellitic diimides (VII, R_1 = H) as shown in Scheme IV.

N-Substituted 1-carbamoyl-2-carboxybenzene-4,5-dicarboxylic anhydrides (III) are prepared by treatment of pyromellitic dianhydride (I) with exactly 1 equiv of ammonia or an amine. Cyclization of compound III afforded the versatile intermediates, pyromellitic N-substituted imide anhydrides (V). These cyclizations were accomplished with either excess thionyl chloride (method A; see Experimental Section for pyromellitic imide anhydride, V, R_1 = H), which gave yields 90% or better, or via the Vilsmeier reagent (method B; see Experimental Section for pyromellitic *N*-methylimide anhydride, V, $R_1 = CH_3$), which gave lower yields (50–60%) but avoided handling hazardous quantities of thionyl chloride for large-scale preparations. It is essential to remove all traces of thionyl chloride and HCl; otherwise the amine in the next step forms a salt and becomes unavailable for reaction. Con-

version of the imide anhydride (V) to the diimide VII by treatment with another amine was accomplished in one step in DMF by gradual application of heat as described for the N-monosubstituted unsymmetrical pyromellitic *N*-(2-hydroxyethyl)diimide (**43**, $R_1 = CH_2CH_2OH$, $R_2 = H$) and for the N,N'-disubstituted unsymmetrical pyromellitic *N*-(2-hydroxyethyl)-*N'*-methyldiimide (**63**, $R_1 = CH_3$, $R_2 = CH_2CH_2OH$). By the use of one or the other of these procedures, pyromellitic diimides having a wide variety of N substituents were synthesized for testing (Fisher et al., 1980; Bochis et al., 1981).

EXPERIMENTAL SECTION

Syntheses. Melting points were measured with a Thomas-Hoover capillary melting point apparatus and are uncorrected. Structural assignments are supported by IR, NMR, and, where helpful, mass spectra. Infrared spectra were obtained from a Nujol mull with a Perkin-Elmer 137 IR spectrometer. A Varian T60A NMR spectrometer and an LKB Model 9000 mass spectrometer were used. Elemental analyses were obtained from the microanalytical laboratory of Merck and are within ±0.4% of theory. Starting materials were commercially available.

Pyromellitic N,N'-Bis(hydroxymethyl)diimide (**11**). NaOH solution, 0.60 mL of 2.5 N, was added to a rapidly stirred mixture of powdered pyromellitic diimide (1), 30.0 g (0.14 mol), and 60 mL of 36% formaldehyde in 300 mL of H_2O. The mixture was heated on a steam bath for 3 h and then cooled in ice. The insoluble product was filtered, rinsed thoroughly with cold H_2O, cold MeOH, and cold Et_2O, and dried in vacuo at 100 °C, furnishing 27.9 g (72%) of white powder: mp >300 °C; IR (Nujol) 3520 (OH), 1770, 1710 (imide C=O) cm^{-1}; ^1H NMR (Me_2SO-d_6) δ 8.30 (s, 2 H, ArH), 6.52 (t, J = 7.0 Hz, 2 H, OH), 5.08 (d, J = 7.0 Hz, 4 H, NCH_2O).

1-Carbamoyl-2-carboxybenzene-4,5-dicarboxylic Anhydride (*Structure III*, $R_1 = H$). During a 75-min period, 25.0 g (1.47 mol) of anhydrous NH_3 was delivered over the surface of a vigorously stirred mixture of 95% pyromellitic dianhydride (I), 321.4 g (1.40 mol), in 3.8 L of cold (5–10 °C) dry acetone. The ice bath was removed and the mixture was stirred at ambient temperature (21 °C) for 60 min longer. The insolubles were filtered and rinsed with acetone. The combined filtrates were concentrated in vacuo with warming at <50 °C to a slurry. Dry EtOAc, 1.14 L, was added and the suspension stirred at reflux for 45 min. The product was filtered, rinsed with hot EtOAc, and dried in vacuo at 55 °C, affording 230.7 g (64%) of white powder: IR (Nujol) 3440, 3300 (NH_2), 2580–2450 (OH), 1840, 1775 (O=COC=O), 1680 (acid C=O), 1630, 1540 ($CONH_2$) cm^{-1}; ^1H NMR (Me_2SO-d_6) δ 8.30, 8.22 (2 s, 2 H, ArH).

Pyromellitic Imide Anhydride (*Structure V*, $R_1 = H$). Thionyl chloride, 13.6 mL (0.19 mol), was added dropwise at a rate to maintain an exotherm of 90–98 °C (ca. 35 min) to a solution of 1-carbamoyl-2-carboxybenzene-4,5-dicarboxylic anhydride, 40.0 g (0.17 mol), in 170 mL of warm (55 °C) dry DMF. The mixture was heated at 90 °C for 35 min longer, and then 170 mL of dry toluene was added over 45 min as the temperature decreased. Stirring was continued for 60 min at ambient temperature. The product was filtered, rinsed with DMF–toluene (1:3) and with dry hexane, and dried in vacuo at 90 °C, furnishing 22.2 g (60%) of fluffy powder: IR (Nujol) 3200 (NH), 1860, 1780 (O=COC=O), 1700 (imide C=O) cm^{-1}; ^1H NMR (dry THF) δ 8.43 (s, ArH).

Pyromellitic N-Methyldiimide (**26**). Methylamine, 9.3 mL (0.21 mol), was delivered as a gas into a stirred suspension of pyromellitic imide anhydride, 43.4 g (0.20 mol), in 600 mL of dry DMF. The mixture was stirred at room temperature (22 °C) for 1 h, at 50 °C for 30 min, at 75 °C for 30 min, and at 150 °C for 1 h. About 400 mL was removed by distillation. The concentrate was diluted with 3 volumes of EtOH and cooled in ice. The crystals were filtered, rinsed with cold EtOH and with Et_2O, and then recrystallized from DMF, furnishing 32.2 g (70%); mp >300 °C; IR (Nujol) 3320 (NH), 1770, 1720, 1690 (imide C=O) cm^{-1}; ^1H NMR (Me_2SO-d_6) δ 8.13 (s, 2 H, ArH), 3.14 (s, 3 H, CH_3N).

Pyromellitic N-(2-Hydroxyethyl)diimide (**43**). Pyromellitic imide anhydride, 21.7 g (0.10 mol), was added with stirring to a solution of 95% ethanolamine, 6.4 mL (0.10 mol), in 100 mL of dry DMF at room temperature (23 °C). The mixture was stirred 30 min and then heated at 50 °C for 30 min, at 75 °C for 30 min, and at 150 °C for 45 min and then cooled in ice and diluted with 200 mL of EtOH. The crystals were filtered and rinsed with cold EtOH and with Et_2O, furnishing 20.8 g (78%): mp 274–275 °C. Recrystallization from DMF gave 13.4 g: mp 279–280 °C; IR (Nujol) 3400 (NH), 1770, 1700 (imide C=O) cm^{-1}; ^1H NMR (Me_2SO-d_6) δ 8.10 (s, 2 H, ArH), 3.67 (s, 2 H, CH_3).

Pyromellitic N-[(2-Methylsulfinyl)ethyl]diimide (**54**). *m*-Chloroperoxybenzoic acid (85%), 5.60 g (27 mmol), in 250 mL of CH_2Cl_2 was added dropwise to a solution of pyromellitic *N*-(2-methylthio)ethyldiimide (**53**), 8.00 g (27 mmol), in 700 mL of MeOH–CH_2Cl_2 (1:9) with stirring at room temperature (23 °C). Mixing was continued for 60 min longer and then the solution was cooled in ice. The crystals were filtered, rinsed with cold CH_2Cl_2, and dried in vacuo, providing 8.00 g (95%): mp 268–269 °C; IR (Nujol) 2710 (NH), 1780, 1720 (imide C=O) cm^{-1}; ^1H NMR (Me_2SO-d_6) δ 8.08 (s, 2 H, ArH), 4.06 (t, J = 6.4 Hz), 3.10 (m), 2.62 (s, 3 H, CH_3SO). Recrystallization from CH_2Cl_2–MeOH gave mp 280–281 °C.

Pyromellitic N-[(2-Methylsulfonyl)ethyl]diimide (**55**). *m*-Chloroperbenzoic acid (85%), 14.5 g (71 mmol), was added to a suspension of pyromellitic *N*-(methylthio)-ethyldiimide (**53**), 8.20 g (28 mmol), in 960 mL of glacial HOAc with stirring at room temperature. The mixture was heated at 100 °C for 4 h and then cooled in ice. The crystals were filtered and rinsed with cold CH_2Cl_2, furnishing 7.80 g (86%): mp 298–299 °C; IR (Nujol) 1760, 1700 (imide C=O), 1270, 1120 (SO_2) cm^{-1}; ^1H NMR (Me_2SO-d_6 plus DCl) δ 8.37 (s, ArH), 4.31 (t, J = 5.6 Hz), 3.77 (t, J = 5.6 Hz), 3.30 (s, CH_3SO_2).

Pyromellitic N-Propionyldiimide (**61**). Powdered pyromellitic diimide (**1**), 29.0 g (0.134 mol), was stirred vigorously in a solution of propionic anhydride, 19.0 mL (0.147 mol), in 1.2 L of pyridine at room temperature (23 °C) for 90 min and then stood overnight. After the mixture was cooled in ice, the crystals were filtered and rinsed with cold pyridine, cold MeOH, and cold Et_2O, furnishing 20.0 g (73%): mp >300 °C; MS m/e 272 (M^+); ^1H NMR (Me_2SO-d_6) δ 7.19 (s, ArH), 3.00 (q, J = 7.4 Hz, $-CH_2C=O$), 1.14 (t, J = 7.4 Hz, CH_3). The product was recrystallized from CH_2Cl_2–MeOH (9:1).

1-(Methylcarbamoyl)-2-carboxybenzene-4,5-dicarboxylic Anhydride (*Structure III*, $R_1 = CH_3$). A solution of 40% aqueous methylamine, 18.1 g (0.23 mol), in 100 mL of acetone was added over a 20-min period to a stirred suspension of 85% pyromellitic dianhydride (I), 60.1 g (0.23 mol), in 800 mL of acetone at 10 °C. Insolubles were filtered and rinsed with a small portion of acetone. The combined acetone filtrates were evaporated in vacuo, and the residue was triturated with 1.0 L of refluxing EtOAc. The product was filtered and rinsed once with hot EtOAc, furnishing 40.6 g (71%): mp 266–268 °C; IR (Nujol) 3350

Table I. Components of Ration

component	% by weight
alfalfa (17% protein)	50.000
animal fat	2.500
wheat bran	10.000
ground corn	32.125
soybean oil meal (48% protein)	5.000
vitamins ADE[a]	0.250
trace minerals, Z-3	0.100
Ethoxyquin, 66.67%	0.025

[a] Supplied 2500 IU of A, 2500 IU of D, and 4 IU of E per lb of ration.

(NH), 1850, 1785 (O=COC=O), 2630, 1725 (CO_2H), 1630, 1600 (CONH) cm^{-1}; ^1H NMR (Me_2SO-d_6) δ 8.07, 8.22 (s, 2 H, ArH), 2.77 (d, J = 4.4 Hz, 3 H, CH_3N).

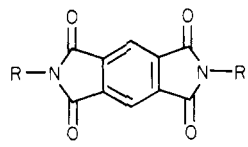
Pyromellitic N-Methylimide Anhydride (V, $R_1 = CH_3$). 1-(Methylcarbamoyl)-2-carboxybenzene-4,5-dicarboxylic anhydride, 39.0 g, in 300 mL of thionyl chloride was stirred at reflux for 4 h. The mixture was cooled and diluted with 300 mL of hexane. The product was filtered, rinsed with hexane, and dried in vacuo at 60 °C over reagent NaOH pellets, providing 32.6 (90%): mp 265–267 °C; IR (Nujol) 1840, 1770 (O=COC=O), 1700 (imide C=O) cm^{-1}; ^1H NMR (Me_2SO-d_6) δ 8.45 (s, 2 H, ArH), 3.18 (s, 3 H, CH_3N).

Pyromellitic N-(Hydroxymethyl)-N′-methyldiimide (**62**). Pyromellitic *N*-methyldiimide (**26**), 2.30 g (10 mmol), was added with vigorous stirring at room temperature (23 °C) to a solution of 36% formaldehyde, 5 mL, in 30 mL of H_2O under N_2 followed by addition of 10 drops of 2.5 NaOH. The mixture was heated at 95 °C for 6 h and then cooled in ice. The product was filtered and rinsed with cold H_2O and with Et_2O, furnishing 1.98 g (76%): mp >300 °C; IR (Nujol) 3490 (OH), 1770, 1700 (imide C=O) cm^{-1}; ^1H NMR (Me_2SO-d_6) δ 8.35 (s, ArH), 5.21 (d, J = 7.0 Hz, NCH_2O), 3.27 (s, CH_3N). The product was recrystallized from DMF–H_2O.

Pyromellitic N-(2-Hydroxyethyl)-N′-methyldiimide (**63**). Pyromellitic *N*-methylimide anhydride, 4.62 g (20 mmol), was added with stirring at room temperature (23 °C) to a solution of ethanolamine, 1.32 mL (22 mmol), in 20 mL of DMF under N_2. The mixture was stirred at ambient temperature for 30 min, at 50 °C for 30 min, at 100 °C for 30 min, and at 150 °C for 1 h and then cooled and diluted with 80 mL of ethanol. The product was filtered and rinsed with cold EtOH and then with Et_2O, affording 4.3 g (78%): mp 271–274 °C; IR (Nujol) 3320 (OH), 1770, 1710 (imide C=O) cm^{-1}; ^1H NMR (Me_2SO-d_6) δ 8.15 (s, ArH), 3.68 (m, OCH_2CH_2N), 3.08 (s, CH_3N). The product was recrystallized from DMF–EtOH.

Pyromellitic N-Methyl-N′-[(2-methylsulfinyl)ethyl]-diimide (**67**). *m*-Chloroperbenzoic acid (85%), 1.02 g (5.0 mmol), in 45 mL of CH_2Cl_2 was added dropwise (45 min) to a solution of pyromellitic *N*-methyl-*N*′-[(2-methylthio)ethyl]diimide (**66**), 1.52 g (5.0 mmol), in 135 mL of MeOH–CH_2Cl_2 (1:9). Solution stirred 1 h longer and then concentrated in vacuo to a small volume. The concentrate was diluted with 2 volumes of Et_2O and chilled in ice. The product was filtered and rinsed with cold Et_2O, yielding 1.20 g (75%): mp 231–233 °C: IR (Nujol) 1050 (SO) cm^{-1};

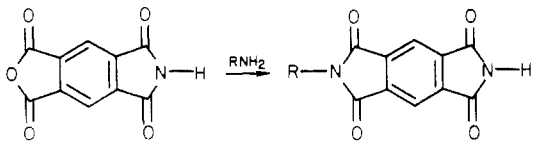
Table II. In Vitro Methane Inhibition[a] of Symmetrical Pyromellitic N,N′-Disubstituted Diimides



compd no.	R	mp, °C	recrystn solvent[b]	yield,[c] %	formula	anal[d]	CH_4 ID_{50},[e] μg/mL
1	H	435[f,g]	A	69[h]	$C_{10}H_4N_2O_4$	CHN	0.75
2	CH_3	378[f,i]	A	75	$C_{12}H_8N_2O_4$	CHN	6.0
3	CH_2CH_3	275-276	B	83	$C_{14}H_{12}N_2O_4$	CHN	10.0
4	$CH_2CH_2CH_3$	239-240	B	90	$C_{16}H_{16}N_2O_4$	CHN	>250
5	$CH_2CH=CH_2$	293-294	B	79	$C_{12}H_{12}N_2O_4$	CHN	7.0
6	$CH_2C(CH_3)=CH_2$	247-248	B	84	$C_{18}H_{16}N_2O_4$	CHN	>250
7	C_6H_{11}	>310	B	83	$C_{16}H_{24}N_2O_4$	CHN	>250
8	C_6H_5[j]	>300	C	64	$C_{22}H_{12}N_2O_4$	CHN	>250
9	$CH_2C_6H_5$	299-300	B	95	$C_{24}H_{16}N_2O_4$	CHN	>250
10	$CH_2CH_2C_6H_5$	>300	B	77	$C_{26}H_{20}N_2O_4$	CHN	>250
11	CH_2OH	>300	D	72[k]	$C_{12}H_8N_2O_6$	CHN	0.75
12	$(CH_2)_2OH$	280-281[l]	D	75	$C_{14}H_{12}N_2O_6$	CHN	1.0
13	$(CH_2)_3OH$	242-243	D	79	$C_{16}H_{16}N_2O_6$	CHN	7.0
14	$(CH_2)_4OH$	218-219.5	E	56	$C_{18}H_{20}N_2O_6$	CHN	5
15	$(CH_2)_5OH$	208-209	E	41	$C_{20}H_{24}N_2O_6$	CHN	20.0
16	$(CH_2)_6OH$	154-155	F	27	$C_{22}H_{28}N_2O_6$	CHN	>20
17	$CH_2C(OH)HC_6H_5$	>300	A	76	$C_{26}H_{20}N_2O_6$	CHN	>250
18	$(CH_2)_2O(CH_2)_2OH$	193-194	G	74	$C_{18}H_{20}N_2O_8$	CHN	50
19	$(CH_2)_2S(CH_2)_2OH$	197-198.5	H	78	$C_{18}H_{20}N_2O_6S$	CHNS	15
20	$(CH_2)_2N(CH_2)_2OH$	131-132.5	F	56	$C_{24}H_{34}N_4O_8$	CHN	>250
21	$(CH_2)_2CO_2H$	>300[m]	A	94	$C_{16}H_{12}N_2O_8$	CHN	>250
22	$(CH_2)_2OCH_3$	177-178	B	48	$C_{16}H_{16}N_2O_6$	CHN	>250
23	$(CH_2)_2SCH_3$	233-234	B	81	$C_{16}H_{16}N_2O_4S_2$	CHNS	>250
24	$(CH_2)_2SH$[n]	232-233	H	77	$C_{14}H_{12}N_2O_4S_2$	CHNS	>250
25	$N(CH_3)_2$	293-294	B	72	$C_{14}H_{14}N_4O_4$	CHN	6.0

[a] For details see Experimental Section, Test Procedures. [b] A = DMF; B = CH_2Cl_2-hexane; C = Me_2SO; D = DMF-H_2O; E = CH_2Cl_2-Et_2O; F = MeOH-Et_2O; G = MeOH; H = CH_2Cl_2. [c] Unless otherwise noted, yields refer to the method shown in Scheme II. For experimental details, see Gitis et al. (1966). [d] Elemental analyses; see Experimental Section, Syntheses. [e] Dose level that inhibited methane production to 50% of the control. [f] Determined by differential thermal analysis. [g] Lit. mp 440 °C (Meyer and Steiner, 1914). [h] Method of Ibing and Neubold (1972) using pyromellitic acid and formamide. [i] Lit. mp 370 °C (Meyer and Steiner, 1914). [j] Meyer and Steiner (1914). [k] Formylation of 1; see Syntheses. [l] Lit. mp 271 °C (Gitis et al., 1966). [m] Lit. mp 329-330 °C (Gitis et al., 1966). [n] Free base of 2-aminoethanethiol hydrochloride was liberated in situ by using triethylamine.

Table III. In Vitro Methane Inhibition[a] of Unsymmetrical Pyromellitic N-Monosubstituted Diimides



compd no.	R	mp, °C	recrystn solvent[b]	yield,[c] %	formula	anal.[d]	CH_4 ID_{50},[e] μg/mL
26	CH_3	>300	A	70	$C_{11}H_6N_2O_4$	CHN	0.50
27	CH_2CH_3	331-332	B	45	$C_{12}H_8N_2O_4$	CHN	1.0
28	$(CH_2)_2CH_3$	260-261	B	41	$C_{13}H_{10}N_2O_4$	CHN	0.75
29	$(CH_2)_3CH_3$	257-258	B	62	$C_{14}H_{12}N_2O_4$	CHN	1.0
30	$(CH_2)_4CH_3$	255-256	B	45	$C_{15}H_{14}N_2O_4$	CHN	4.0
31	$(CH_2)_5CH_3$	243.5-245	B	35	$C_{16}H_{16}N_2O_4$	CHN	8.0
32	$(CH_2)_6CH_3$	241-242	B	36	$C_{17}H_{18}N_2O_4$	CHN	>250
33	$CH(CH_3)_2$	274-276	B	16	$C_{13}H_{10}N_2O_4$	CHN	0.75
34	$CH_2CH{=}CH_2$	248.5-249.5	B	54	$C_{13}H_8N_2O_4$	CHN	2.0
35	$CH_2C{\equiv}CH$	288-289	C	62	$C_{13}H_6N_2O_4$	CHN	3.0
36	cyclopropyl	>300	A	78	$C_{13}H_8N_2O_4$	CHN	1.0
37	C_6H_{11}	305.5-307	A	58	$C_{16}H_{14}N_2O_4$	CHN	5.0
38	C_6H_5	>360	A	40	$C_{16}H_8N_2O_4$	CHN	>250
39	$CH_2C_6H_5$	286-287	A	50	$C_{17}H_{10}N_2O_4$	CHN	2.0
40	C_6H_4OH (*p*)	>300	A	74	$C_{16}H_8N_2O_5$	CHN	3.0
41	$C_6H_4NO_2$ (*p*)	>300	A	59	$C_{16}H_7N_3O_6$	CHN	4.0
42	$C_6H_4SCH_3$ (*p*)	>300	A	79	$C_{17}H_{10}N_2O_4S$	CHNS	>250
43	$(CH_2)_2OH$	279-280	C	78	$C_{12}H_8N_2O_5$	CHN	1.0
44	$(CH_2)_3OH$	258-260	A	66	$C_{13}H_{10}N_2O_5$	CHN	0.75
45	$(CH_2)_4OH$	232-233	A	46	$C_{14}H_{12}N_2O_5$	CHN	0.50
46	$(CH_2)_6OH$	218-219.5	B	63	$C_{16}H_{16}N_2O_5$	CHN	0.75
47	$C(C_2H_5)HCH_2OH$	204-205	B	58	$C_{14}H_{12}N_2O_5$	CHN	0.75
48	$(CH_2)_2O(CH_2)_2OH$	232-233.5	B	80	$C_{14}H_{12}N_2O_6$	CHN	0.50
49	$(CH_2)_2S(CH_2)_2OH$	215-216	B	51	$C_{14}H_{12}N_2O_5S$	CHNS	0.50
50	$CH_2CH(OH)CH_2OH$	259-261	B	58	$C_{13}H_{10}N_2O_6$	CHN	8.0
51	$(CH_2)_2OCH_3$	250-251	B	47	$C_{13}H_{10}N_2O_5$	CHN	1.5
52	$(CH_2)_2SH$	>300	A	78[f]	$C_{12}H_8N_2O_4S$	CHNS	2.0
53	$(CH_2)_2SCH_3$	253-254	D	82	$C_{13}H_{10}N_2O_4S$	CHNS	0.75
54	$(CH_2)_2SOCH_3$	280-281	D	95[g]	$C_{13}H_{10}N_2O_5S$	CHNS	0.50
55	$(CH_2)_2SO_2CH_3$	298-299	A	86[g]	$C_{13}H_{10}N_2O_6S$	CHNS	2.0
56	CH_2CO_2H	>300	A	46	$C_{12}H_6N_2O_6$	CHN	15.0
57	$(CH_2)_2CO_2H$	>300	A	66	$C_{13}H_8N_2O_6$	CHN	3.0
58	CH_2CONH_2	>300	A	70[f]	$C_{12}H_7N_3O_5$	CHN	2.0
59	CH_2CN	307-309	A	22[f]	$C_{12}H_5N_3O_4$	CHN	20.0
60	$(CH_2)_2N(CH_3)_2$	255-257	B	48	$C_{14}H_{13}N_3O_4$	CHN	50.0
61	$COCH_2CH_3$	>300	D	54[h]	$C_{13}H_8N_2O_5$	CHN	1.0

[a] See footnote *a*, Table II. [b] A = DMF-EtOH; B = EtOH; C = DMF; D = CH_2Cl_2-MeOH. [c] Unless otherwise noted, yields refer to the last reaction of Scheme IV utilizing the intermediate pyromellitic imide anhydride as shown above and as described for compound **43** under Syntheses. [d] See footnote *d*, Table II. [e] See footnote *e*, Table II. [f] The appropriate amine hydrochloride was converted to the free base in situ by using an equivalent of triethylamine. The product was crystallized by addition of H_2O to the reaction mixture in place of ethanol. [g] Oxidation of compound **53**; see Syntheses. [h] Acetylation of compound 1; see Syntheses.

^1H NMR (Me_2SO-d_6) δ 2.58 (s, $CH_3S{=}O$). The product was recrystallized from CH_2Cl_2–Et_2O.

Pyromellitic N-(Acetoxymethyl)-N'-methyldiimide (**68**). Pyromellitic *N*-(hydroxymethyl)-*N'*-methyldiimide (**62**), 1.30 g (5.0 mmol), was added to a solution of acetic anhydride, 2.4 mL (25 mmol), in 20 mL of pyridine and the mixture heated at 95 °C for 2 h. The resulting solution was concentrated, diluted with MeOH, and cooled in ice. The product was filtered and rinsed with cold MeOH and with cold ether, furnishing 1.07 g (70%): mp 153–154 °C; ^1H NMR (Me_2SO-d_6) δ 8.25 (s, ArH), 5.04 (s, NCH_2O), 3.12 (s, CH_3N), 2.05 (s, $CH_3C{=}O$). The product was recrystallized from DMF–EtOH.

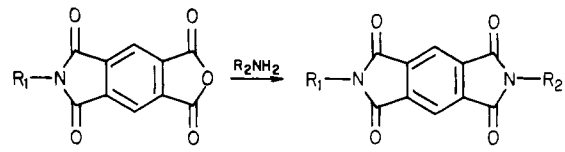
Test Procedures: In Vitro Rumen Assay. A 16 × 100 mm screw-cap tube fitted with a Wheaton slotted gray butyl stopper was used for the fermentation vessel. A hole was drilled through the screw cap for sampling gas production. Each tube contained 0.5 mL of buffer (Halliwell, 1957), 100 mg of ration (see Table I) ground to $^1/_2$-mm mesh, and 3.5 mL of sheep rumen fluid strained through four layers of cheesecloth. Compounds were diluted with Super-Cel and added to each tube at the appropriate dose level. During addition of rumen fluid, the tubes were gassed with 97% N_2–3% H_2 passed through a Deoxo catalytic purifier to remove O_2. Samples were incubated at 39 °C for 18 h in a water bath shaker at 250 rpm. A sample of rumen fluid was held frozen for use as the 0-h control.

Gas ratios were read in a Model 5 Fisher gas partitioner and compared to that of a standard gas mixture. The fermentation liquid was acidified with 4 mL of 10% metaphosphoric acid and centrifuged at 9000 rpm for 10 min. One microliter of the supernatant was used for gas–liquid chromatography for VFA determination by the method of Erwin et al. (1961), using Hewlett-Packard gas chromatographs 402 and 7610A with a column packing of 10% neopentyl glycol succinate–196 H_3PO_4 on 80–100-mesh Chromasorb WHP as supplied by Applied Science Laboratories. CH_4 ID_{50} and VFA data are shown in Tables II–V.

RESULTS AND DISCUSSION

Tables II–IV list the dose levels of the diimides in micrograms per milliliter that inhibited methane production

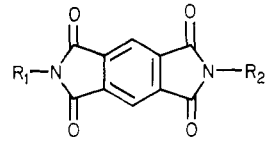
Table IV. In Vitro Methane Inhibition[a] of Unsymmetrical Pyromellitic N,N'-Disubstituted Diimides



compd no.	R_1	R_2	mp, °C	yield,[b] %	formula	anal.[c]	CH_4ID_{50},[d] μg/mL
62	CH_3	CH_2OH	>300	76[e]	$C_{12}H_8N_2O_5$	CHN	0.5
63	CH_3	$(CH_2)_2OH$	271-274	78	$C_{13}H_{10}N_2O_5$	CHN	5.0
64	CH_3	$(CH_2)_3OH$	235-236	86	$C_{14}H_{12}N_2O_5$	CHN	4.0
65	CH_3	CH_2CH_3	285-287	38	$C_{13}H_{10}N_2O_4$	CHN	2.0
66	CH_3	$(CH_2)_2SCH_3$	208-209	90	$C_{14}H_{12}N_2O_4S$	CHNS	7.0
67	CH_3	$(CH_2)_2SOCH_3$	231-233	75[f]	$C_{14}H_{12}N_2O_5S$	CHNS	4.0
68	CH_3	CH_2OCOCH_3	153-154	70[g]	$C_{14}H_{10}N_2O_6$	CHNS	1.5
69	C_2H_5	CH_2OH	>300	75[h]	$C_{13}H_{10}N_2O_5$	CHN	0.75
70	CH_2OH	$(CH_2)_2OH$	273-274	36[i]	$C_{13}H_{10}N_2O_6$	CHN	2.0
71	CH_2OH	$CH_2C_6H_5$	284-285	65[j]	$C_{18}H_{12}N_2O_5$	CHN	4.0
72	$COCH_3$	$(CH_2)_2OCOCH_3$	176-178	73[k]	$C_{16}H_{12}N_2O_7$	CHN	1.5

[a] See footnote *a*, Table II. [b] Unless otherwise noted, yields refer to the last step of Scheme IV utilizing pyromellitic N-substituted imide anhydride intermediates as shown above and as described for compound **63** under Syntheses. [c] See footnote *d*, Table II. [d] See footnote *e*, Table II. [e] Formylation of compound **26**; see Syntheses. [f] Oxidation of compound **66**; see Syntheses. [g] Acetylation of compound **62**; see Syntheses. [h] Formylation of compound **27** by the method for compound **62**. [i] Formylation of compound **43** by the method for compound **62**. [j] Formylation of compound **39** by the method for compound **62**. [k] Acetylation of compound **43** by the method for compound **68**.

Table V. In Vitro Rumen Volatile Fatty Acid Composition[a] for the Most Potent Pyromellitic Diimides



compd no.	R_1	R_2	CH_4 ID_{50},[b] μg/mL	VFA produced, molar %				acetic/propionic ratio
				acetic	propionic	butyric	valeric	
control				51	33	12	4	1.55
1	H	H	0.75	36 (-29)[c]	35 (+6)	24 (+100)	5 (+25)	1.03
11	CH_2OH	CH_2OH	0.75	40 (-22)	36 (+9)	20 (+67)	5 (+25)	1.11
12	$(CH_2)_2OH$	$(CH_2)_2OH$	1.0	41 (-20)	41 (+24)	12 (0)	6 (+50)	1.00
26	CH_3	H	0.50	34 (-33)	38 (+15)	23 (+92)	6 (+50)	0.89
43	CH_2OH	H	1.0	35 (-31)	37 (+12)	23 (+92)	6 (+50)	0.95
48	$(CH_2)_2O(CH_2)_2OH$	H	0.50	31 (-39)	40 (+21)	23 (+92)	6 (+50)	0.78
49	$(CH_2)_2S(CH_2)_2OH$	H	0.50	33 (-35)	37 (+12)	22 (+83)	7 (+75)	0.89
53	$(CH_2)_2SCH_3$	H	0.75	32 (-37)	38 (+15)	23 (+92)	6 (+50)	0.84
54	$(CH_2)_2SOCH_3$	H	0.50	25 (-51)	40 (+21)	24 (+100)	11 (+175)	0.63
55	$(CH_2)SO_2CH_3$	H	2.0	21 (-59)	42 (+27)	26 (+117)	11 (+175)	0.50
62	CH_3	CH_2OH	0.50	33 (-35)	39 (+18)	20 (+67)	8 (+100)	0.85
69	C_2H_5	CH_2OH	0.75	31 (-39)	40 (+21)	21 (+75)	8 (+100)	0.78
70	CH_2OH	$(CH_2)_2OH$	2.0	27 (-47)	39 (+18)	26 (+117)	9 (+125)	0.69

[a] See footnote *a*, Table II. [b] See footnote *e*, Table II. [c] Data in parentheses refer to percent change from the control.

in the in vitro rumen fermentation to 50% of the control, CH_4 ID_{50}. The VFA compositions for some of the most potent compounds are listed in Table V and are given as (1) molar percent of VFA produced, (2) percent change from the control and (3) as the acetic/propionic ratio, all at the CH_4 ID_{50} level.

The most potent compounds in the symmetrical series, shown in Table II, were pyromellitic diimide (**1**) and pyromellitic *N,N'*-bis(2-hydroxyethyl)diimide (**12**). Pyromellitic *N,N'*-bis(hydroxymethyl)diimide (**11**) appeared to have similar activity, which may have been due to loss of formaldehyde in the rumen fluid reflecting the activity of the starting material.

The dimethyl (**2**) and diethyl (**3**) derivatives were also highly active, but activity was abruptly lost with dipropyl (**4**) substitution. Hydroxyl groups restored activity to otherwise inactive compounds. For example, **13**, the dihydroxy derivative of **4**, was active at 7 μg/mL where **4** was inactive at 250 μg/mL. Cycloalkyl, aryl, and aralkyl substitution produced inactive compounds.

The monosubstituted pyromellitic diimides shown in Table III were generally highly active through a wide range of substitution, although activity was lost in the heptyl (**31**) and phenyl (**38**) derivatives. Alkyl substituents such as methyl, ethyl, propyl, isopropyl, and butyl were tolerated with virtually no change in activity.

Hydroxyalkyl substituents as in compounds **43–49** caused no reduction in activity. Even the cyclohexyl (**37**) and benzyl (**39**) derivatives were highly effective whereas the corresponding disubstituted analogues (**7**) and **9**) were inactive at 250 μg/mL. A similar finding was also true for a series of alkyl derivatives substituted with OCH_3, SH, SCH_3, and CO_2H, compounds **51**, **52**, **53**, and **57**, respectively, as compared with the corresponding disubstituted compounds **22**, **24**, **23**, and **21**.

Synthesis of unsymmetrically disubstituted pyromellitic diimides was restricted to those substituents showing good activity in the other series. The compounds prepared are

listed in Table IV. The activities of **62**, **69**, **70**, and **71** again probably reflect loss of formaldehyde. The other compounds were active in the range of 2–7 μg/mL as expected.

The percent changes in the VFA compositions listed in Table V show a marked decrease in acetate, a range of −20 to −59%, associated with increases in propionate (+6 to +27%), butyrate (0 to +117%), and valerate (+25 to +175%). The acetic/propionic ratio was reduced from 1.55 for the control to a range of 0.50–1.11. Combined with the high potency of these compounds in the reduction of methane, CH_4 ID_{50} in the range of 0.50–2.0 μg/mL, these results have prompted additional testing of the diimides on the ruminal parameters in sheep. The sheep tests will be reported separately (Baylis et al., 1982).

From all of these data, and also taking into account relative cost and ease of synthesis, pyromellitic diimide (**1**) and pyromellitic *N*-(2-hydroxyethyl)diimide (**43**) were judged to be the most promising compounds for further study and are now undergoing investigation for enhancement of feed efficiency in ruminants.

ACKNOWLEDGMENT

We thank Jack P. Gilbert and Jane T. Wu for analytical data, Herman Flynn and Jack L. Smith for NMR and MS spectra, and Michael A. Penn for valuable technical assistance.

LITERATURE CITED

Baylis, F. P.; Linn, B. O.; Paege, L. M.; Doherty, P. J.; Bochis, R. J.; Waksmunski, F. S.; Kulsa, P.; Fisher, M. H.; Smith, G. E., submitted for publication in *J. Anim. Sci.*, 1982.

Bochis, R. J.; Fisher, M. H.; Linn, B. O. U.S. Patent 4 289 784, 1981.

Chalupa, W. *J. Anim. Sci* **1977**, *46* (3), 585.

Davis, G. V.; Erhart, A. B. *J. Anim. Sci.* **1976**, *43* (1), 1.

Erwin, E. S.; Marco, G. J.; Emery, E. M. *J. Dairy Sci.* **1961**, *44*, 1768.

Fisher, M. H.; Kulsa, P.; Linn, B. O. U.S. Patent 4 212 880, 1980.

Gitis, S. S.; Ivanova, V. M.; Nemleva, S. A.; Seina, Z. N. *J. Org. Chem. USSR (Engl. Transl.)* **1966**, *2* (7), 1261; *Zh. Org. Khim.* **1966**, *2* (7), 1265.

Halliwell, G. *J. Gen. Microbiol.* **1957**, *17*, 153.

Hungate, R. E. In "The Rumen and Its Microbes"; Academic Press: New York, 1966; p 273.

Ibing, G.; Neubold, K. U.S. Patent 3 697 541, 1972.

Leng, R. A. In "Physiology of Digestion and Metabolism in the Ruminant"; Phillipson, A. T., Ed.; Oriel Press: Newcastle upon Tyne, England, 1970; p 410.

Meyer, H.; Steiner, K. *Monatsh. Chem.* **1914**, *35*, 391.

Perry, T. W.; Beeson, W. M.; Mohler, M. T. *J. Anim. Sci.* **1976**, *42* (3), 761.

Potter, E. L.; Cooley, C. O.; Richardson, L. F.; Raun, A. P.; Rathmacher, R. P. *J. Anim. Sci.* **1976a**, *43* (3), 665.

Potter, E. L.; Raun, A. P.; Cooley, C. O.; Rathmacher, R. P.; Richardson, L. F. *J. Anim. Sci* **1976b**, *43* (3), 678.

Raun, A. P.; Cooley, C. O.; Potter, E. L.; Rathmacher, R. P.; Richardson, L. F. *J. Anim. Sci* **1976**, *43* (3), 670.

Richardson, L. F.; Raun, A. P.; Potter, E. L.; Cooley, C. O.; Rathmacher, R. P. *J. Anim. Sci.* **1976**, *43* (3), 657.

Smith, R. K.; Popoff, C. U.S. Patent 3 078 228, 1963.

Wolin, M. J. *Am. J. Clin. Nutr.* **1974**, *27*, 1320.

Received for review January 4, 1982. Revised manuscript received August 2, 1982. Accepted August 30, 1982.

COMMUNICATIONS

Analysis of Sugars in Foods Containing Sodium Chloride by High-Performance Liquid Chromatography

The presence of salt in foods was found to interfere with the analysis of sugars by high-performance liquid chromatography due to chloride ion producing a response with the refractive index detector that was close to that of glucose. The addition of a PIC reagent (tetrabutylammonium phosphate) to the acetonitrile/water mobile phase markedly reduced the retention time of chloride so that it eluted well before any sugar. The PIC reagent also improved the resolution of the sugars.

Analysis of individual sugars by high-performance liquid chromatography (HPLC) using a silica-based weak anion exchange column with acetonitrile/water as the mobile phase and a refractive index detector has been reported for a wide range of foods [e.g., De Vries et al. (1979), Hurst et al. (1979), and Warthesen and Kramer (1979)], has been adopted as an official AOAC method (No. 31.138-145; AOAC, 1980) and is routinely used in many research and quality control laboratories. We have found that for many processed foods an extra peak that did not correspond to any sugar was present and interfered with the estimation of glucose (Figure 1). In this paper we report on a method for removing this interference.

MATERIALS AND METHODS

Sugars were extracted from food with hot 85% v/v methanol, and the volume of the solution was reduced by evaporation and made up to 10 mL with water (Wills et al., 1980). After filtration through a membrane ultrafilter, an aliquot (20 μL) was injected onto a μBondapak/carbohydrate column (Waters Associates) installed in a Waters liquid chromatograph (Model No. ALC/GPC 244) equipped with a 41-MPa pump and U6K injector. The mobile phase was acetonitrile/water (80/20) at 2 mL/min, and column effluents were monitored with a refractive index detector (Waters Associates Model No. R401) at ×8 attenuation. Individual sugars were identified on the basis of their retention times. A range of salts and organic acids was injected onto the column to establish the identity of the nonsugar compound. The addition of the ion pairing reagent, tetrabutylammonium phosphate (PIC A, Waters Associates) to the mobile phase was examined to remove

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