Journal of Chromatography, 143 (1977) 247—257
Biomedical Applications

CHROMBIO. 034

GAS CHROMATOGRAPHY—MASS SPECTROMETRY OF METABOLITES IN HEMODIALYSIS FLUID

TED L. MASIMORE* and HANS VEENING

Department of Chemistry, Bucknell University, Lewisburg, Pa. 17837 (U.S.A.)

W.J.A. VANDENHEUVEL

Merck Sharp & Dohme Research Laboratories, Rahway, N.J. 07065 (U.S.A.)

and

DAVID A. DAYTON

Divine Providence Hospital, Williamsport, Pa. 17701 (U.S.A.)

(Received October 28th, 1976)

SUMMARY

An analytical method has been developed for the separation and identification of several metabolites in used hemodialysis fluid obtained during the treatment of a uremic patient on the artificial kidney. The procedure involves ion exchange, evaporation, and trimethylsilylation; the derivatized components were studied by combined gas chromatography—mass spectrometry.

Twelve compounds were satisfactorily resolved; six were conclusively identified from mass spectral data. The identified components include phosphoric acid, glucopyranurono-(6→1)-lactone, citric acid, D-gluconic acid-δ-lactone, α-D-glucose, and β-D-glucose. A seventh component was tentatively identified as mannonic acid.

INTRODUCTION

Gas chromatography (GC) has been used extensively for the separation of molecular components in physiological fluids [1—3]. Identification of many of these components has been achieved by combined gas chromatography—mass

*Present Address: Wyeth Laboratories, Inc., P.O. Box 861, Paoli, Pa. 19301 (U.S.A.).

spectrometry (GC—MS). Horning et al. [4] have demonstrated that GC—MS can be used to measure a wide variety of components of clinical interest. Thompson and Markey [5] have compared the reproducibility and efficiency of methods of isolation of organic acids from urine by means of a GC—MS—computer system.

This paper describes a GC—MS study of components found in the dialysate obtained during hemodialysis of a patient with chronic renal failure. Patients with chronic renal failure produce little or no urine, and they therefore retain large quantities of water and electrolytes, as well as those waste substances that are normally excreted. These substances, which accumulate in the blood, must be removed periodically by means of hemodialysis. In this process, the excess or waste substances are removed from the bloodstream by dialysis through a cellulose or copolymer semipermeable membrane in an artificial kidney. The aqueous receptor fluid is known as the hemodialysate (or simply dialysate); it is prepared by diluting a commercial concentrate of dissolved electrolytes (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, and acetate) with sufficient water, so that the final volume is 120 l. In the artificial kidney, arterial blood is pumped through the membrane at ca. 200 ml/min. Dialysate flows on the outside of the membrane at ca. 0.3 l/min, its direction of flow being opposed to that of the blood, thus producing a useful concentration gradient. Typical dialysis membranes contain 40-Å pores, and are permeable to substances up to 18,000 molecular weight [6].

It has been shown in a number of studies that the syndrome of uremia is directly attributable to the presence of substances in body fluids at concentrations that are considerably higher than those normally attained in healthy subjects [6—9]. Recently, Bultitude and Newham [8] have reported a method for comparing plasma samples from chronic uremic patients before and after dialysis. They identified a number of abnormal metabolites by use of combined GC—MS of the trimethylsilyated derivatives, and showed that the concentrations of these compounds increased in uremia; however, after dialysis of the patient's blood, the concentrations became about the same as those in plasma from healthy subjects. Low-molecular-weight volatiles have also been extracted from the blood plasma of patients, before and after hemodialysis, and have been separated and identified by GC—MS—computer methods [6].

In much of the work reported thus far, attention has been devoted primarily to the analysis of blood plasma from uremic subjects. It has been generally assumed that used dialysate contains concentrations of metabolites that are too low to be detected; however, recently it has been shown that used hemodialysate can be analyzed efficiently by high-resolution liquid chromatography [10]. In the present investigation, it has been found that used dialysate sampled from the effluent of a multiple-pass artificial kidney can also be analyzed by GC—MS.

MATERIALS AND METHODS

Samples

Several 1-l samples of used hemodialysate (Travenol Laboratories, Deerfield, Ill., U.S.A.) were collected from a female patient on an artificial kidney ca. 3 h

after the start of treatment. The samples were filtered through a 115-ml, 0.20-μm Nalgene membrane filter (Sybron, Rochester, N.Y., U.S.A.). Several drops of reagent-grade chloroform were added to each sample as a bacteriostatic agent, and the samples were then frozen and stored at −15° until ready for use. Blank (or unused) hemodialysate samples were obtained from the aqueous solution of electrolytes, prior to patient treatment, and were treated and stored identically.

Sample preparation

Acidic (and some neutral) compounds were separated from hemodialysate samples by use of DEAE-Sephadex, a weakly basic anion exchanger, (Pharmacia, Piscataway, N.J., U.S.A.) [1]. The ion-exchange bed was prepared in a 25-ml buret, equipped with a small glass wool plug. A slurry of the resin suspended in 1.5 *M* pyridine acetate was poured into the buret until a 1.2 × 10 cm resin bed was produced. The column was first treated with 150 ml of 1.5 *M* pyridine acetate, followed by 200 ml of 0.5 *M* pyridine acetate. A 150-ml dialysate sample was introduced into the column by means of a constant flow device [11]; this was followed by the addition of 150 ml of doubly distilled water. The sample components were eluted with 150 ml of 1.5 *M* pyridine acetate using a flow-rate of 1 ml/min. The eluent was collected and frozen at −15° until ready for further processing.

The thawed sample was evaporated at 25° and 1 mmHg pressure. After the sample was reduced to a viscous fluid (ca. 2.5 h), crystallization occurred. This procedure was continued for an additional 2.5 h in order to remove the last traces of solvent. When drying was complete, the residue was dissolved in 2 ml of absolute methanol and quantitatively transferred to a 3-ml reaction vial (Supelco, Bellefonte, Pa., U.S.A.) for trimethylsilylation. The methanol was next evaporated from the vial under a stream of dry nitrogen at 25°.

Sample derivatization

The components in the dried residue were derivatized by adding 0.3 ml of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Pierce, Rockford, Ill., U.S.A.) from a glass syringe. The mixture was shaken and placed in a sand-bath at 60° for 5 min. The mixture was then shaken again and allowed to stand in the sand-bath for an additional 5 min, whereupon it was cooled to room temperature. Solid residue precipitated from a supernatant liquid layer that contained the dissolved trimethylsilyl (TMS) derivatives. For combined GC—MS experiments, a second portion of the sample was derivatized with N,O-bis-(perdeuterotrimethylsilyl)acetamide (BSA-d_{18}; Merck Sharp & Dohme, Montreal, Canada). This reaction produced the corresponding TMS-d_9 derivatives which facilitated mass spectral interpretation.

Gas chromatography—mass spectrometry

The TMS derivatives were separated on a Perkin-Elmer Model 900 gas chromatograph, equipped with a 12 ft. × 2 mm I.D. glass column packed with 3.3% SE-30 on 100—200 mesh Gas-Chrom Q. Helium was used as the carrier gas at a flow-rate of 71 ml/min and at a column inlet pressure of 80 p.s.i.g. The instrument was equipped with a glass-lined injection port operated at 265°,

and the eluted components were monitored by means of a flame-ionization detector operated at 270°, and fueled with hydrogen at 20 p.s.i.g. and ultra-zero air at 40 p.s.i.g. Samples (1.0—5.0 μl) of the supernatant layer in the reaction vial were injected with a 10-μl syringe. The column was operated isothermally at 100° during the initial 8 min of a run, and was then programmed to 260° at 5°/min. The final temperature was maintained for 10 min. The total time of separation for a typical chromatographic elution was ca. 50 min.

For combined GC—MS studies, an LKB Model 9000 gas chromatograph—mass spectrometer was used. Aliquots (1—5 μl) of the derivatized samples were analyzed using a 10 ft. × 4 mm glass column packed with 3% OV-101 on 80—100 mesh Gas-Chrom P, which was operated as above. Helium was used as a carrier gas at a flow-rate of 30 ml/min. Mass spectrometric conditions included 70 eV ionization potential, 3.5 kV accelerating voltage, 60 μA trap current, and 270° source temperature.

RESULTS

Gas chromatograms were recorded for several blank and used hemodialysate samples for comparison purposes in order to determine the 'background', or control components, in the derivatized commercial hemodialysate concentrate and in the municipal water used for dilution. Gas chromatograms for blank and used hemodialysate samples are shown in Figs. 1 and 2, respectively. It is seen that both yield a number of components eluting between 0 and 10 min, but the patterns are very similar for the first 10 min. Gas chromatograms were also

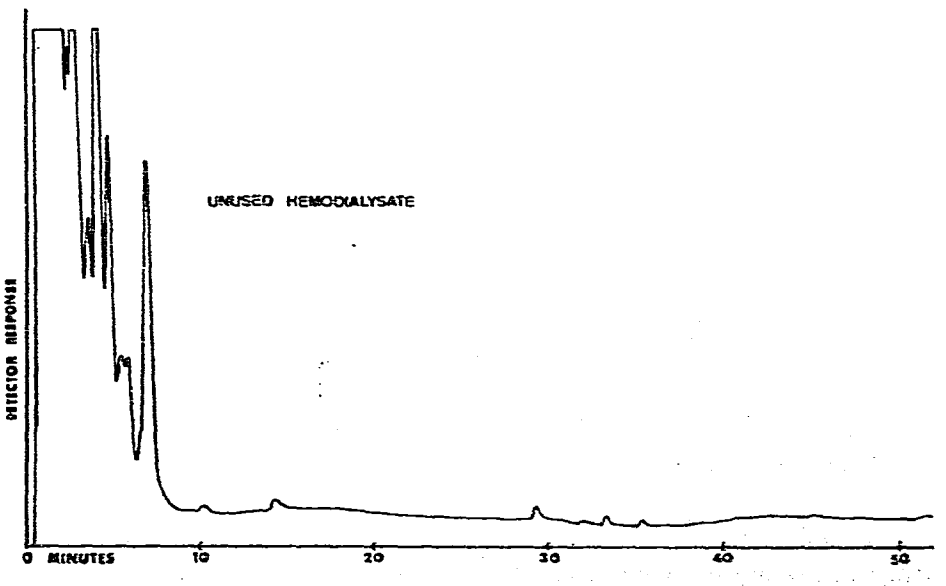


Fig. 1. Gas chromatogram for blank hemodialysate. Sample volume: 1.8 μl. Attenuation: × 80.

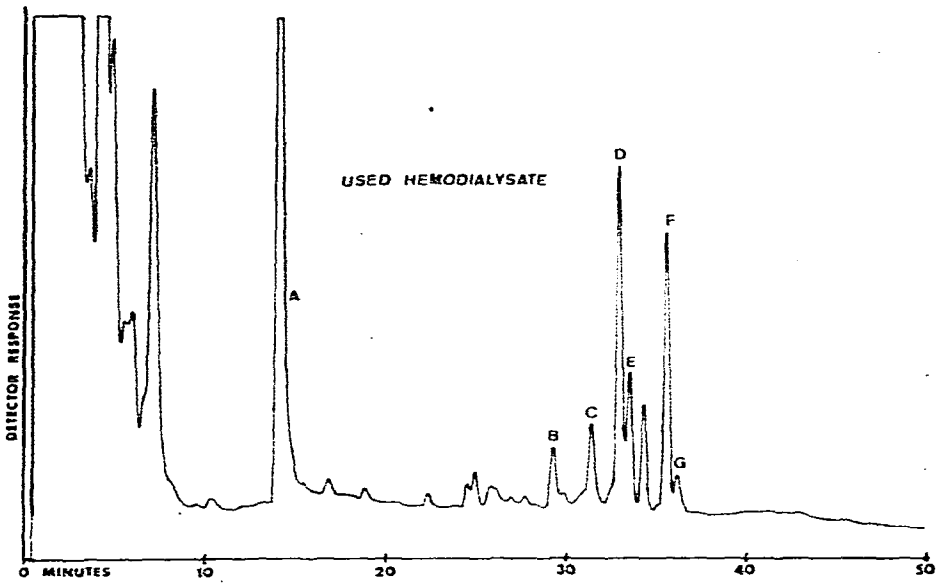


Fig. 2. Gas chromatogram for used hemodialysate derivatized with BSTFA. Sample volume: 2.5 μl. Attenuation: × 80.

recorded for reagent blanks (samples using doubly distilled water in place of hemodialysate); these were essentially identical with the blank hemodialysate samples (Fig. 1); thus no major peaks are due to the reagents used in sample preparation and derivatization. Fig. 2 shows that there are eight major components present in the used hemodialysate sample. It was found that slight variations in peak sizes did occur from run to run for individually processed aliquots of the same used hemodialysate sample; reproducibility of the retention times for the components was acceptable (±3%). The gas chromatogram resulting from a used hemodialysate sample derivatized with BSA-d_{18} is presented in Fig. 3.

Peak identification

Identification of the major components in used hemodialysate was accomplished by use of GC—MS. Mass spectral data were collected for the TMS and TMS-d_9 components, A through G (Fig. 2).

Component A was found to be the tris-TMS derivative of phosphoric acid (MW = 314). The mass spectra obtained for derivatized phosphoric acid were found to be similar to previously published data for the TMS and TMS-d_9 derivatives [12—14]. The MS data for peak A are presented in Table I; they indicate a molecular ion at *m/e* 314 and an intense M − 15 fragment ion. The *m/e* 211 ion is known [12] to be formed by the loss of a TMS group and two methyl radicals from the M^+ ion, followed by rearrangement to produce $(CH_3)_4Si_2PO_4$. Mass spectral data for the deuterium-labeled analogue are also presented in Table I. They are consistent with, and verify, the identity of peak

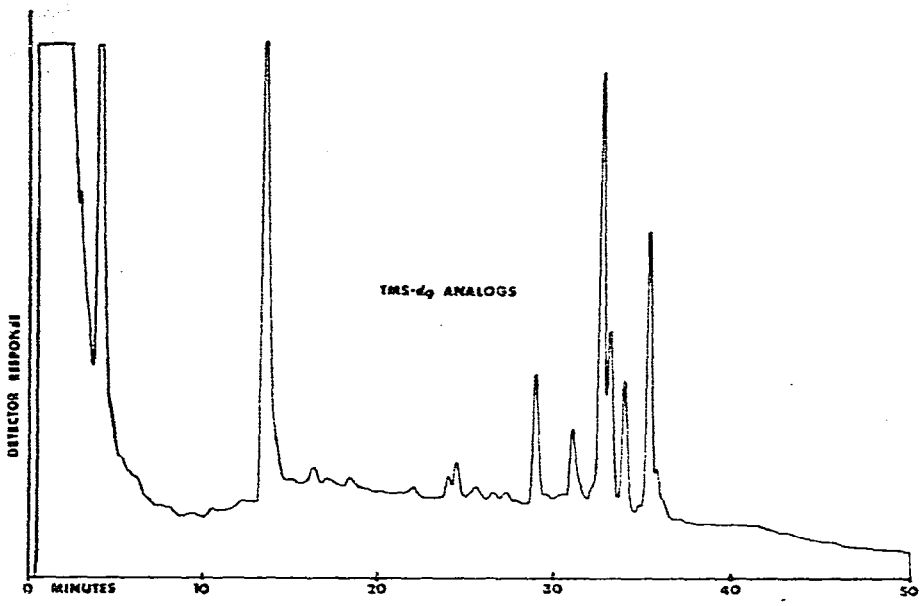


Fig. 3. Gas chromatogram for used hemodialysate derivatized with BSA-d_{18}. Sample volume: 1.4 µl. Attenuation: × 80.

TABLE I

MASS SPECTRAL DATA FOR COMPONENT A

TMS derivative		TMS-d_9 derivative *m/e*	Mass shift
m/e	Rel. int. (%)		
314, M	23	341, M	27
299	100	323	24
283	5	303	20
211	5	223	12
207	8	222	15
147	7	162	15
133	12	142	9
73	71	82	9

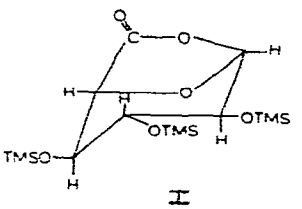
A. For example, the *m/e* TMS-d_9 molecular ion shows a mass change of 27, thus indicating the presence of three silylated positions on the phosphoric acid molecule. The *m/e* 283 ion arises from the loss of a hydrogen atom and two methyl radicals from the molecular ion (M − 31, TMS; M − 38, TMS-d_9). The retention time for derivatized authentic phosphoric acid also agreed exactly with that of component A (Fig. 2).

Component B was identified as the tris-TMS ether of glucopyranurono-(6→1)-lactone (I). The pertinent MS data are presented in Table II.

TABLE II

MASS SPECTRAL DATA FOR COMPONENT B

TMS derivative		TMS-d_9 derivative	Mass shift
m/e	Rel. int. (%)	*m/e*	
392, M	6	419, M	27
377	2	401	24
348	2	375	27
306	7	333	27
305	5	332	27
291	1	315	24
217	47	235	18
204	16	222	18
191	4	209	18
189	3	204	15
147	20	162	15
73	100	82	9



I

The molecular ion was observed at *m/e* 392, with an M − 15 ion at *m/e* 377. Other important signals for component B occurred at *m/e* 305, 306 and 204. The *m/e* 305 and 306 ions are known to be present in the mass spectrum of the 6→1 lactone and absent in the spectrum of the 6→3 isomer [15, 16]. Also, the intense *m/e* 230 ion found in the 6→3 lactone [16] is absent from the mass spectrum for component B. The molecular ion for the TMS-d_9 derivative appeared at *m/e* 419; this mass shift of 27 *m/e* units with respect to the TMS molecular ion provides additional evidence for the tris-TMS structure. GC of trimethylsilylated acyl glucuronides has been found to result in the elution of the trimethylsilylated 6→1 lactone [15]. Interestingly, it has been reported [16] that the 6→1 lactone is also produced during GC of a trimethylsilylated human urinary metabolite of cyproheptadine (Periactin), a drug administered to the patient who underwent hemodialysis.

Component C was identified from GC and MS data (Table III) as the tetra-TMS derivative of citric acid. Comparison of the molecular ion values for the TMS and TMS-d_9 compounds requires a molecular weight of 192 for the parent compound, suggesting C to be citric acid. The mass spectrum obtained for component C was virtually identical to that published by Dalgliesh et al. [17] for the TMS derivative of citric acid, and the GC retention time for component C was found to be identical to that of a sample of trimethylsilylated reference material.

Mass spectral data for component D are summarized in Table IV. The com-

TABLE III

MASS SPECTRAL DATA FOR COMPONENT C

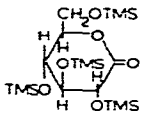
TMS derivative		TMS-d, derivative	Mass shift
m/e	Rel. int. (%)	*m/e*	
480, M	0.6	516, M	36
465	19	498	33
375	20	399	24
363	26	390	27
347	17	371	24
333	2	357	24
319	4	343	24
305	6	332	27
285	5	300	15
273	65	291	18
257	3	272	15
245	5	263	18
231	4	249	18
221	7	242	21
217	7	235	18
211	7	220	9
183	3	192	9
147	51	162	15
73	100	82	9

TABLE IV

MASS SPECTRAL DATA FOR COMPONENT D

TMS derivative		TMS-d, derivative	Mass shift
m/e	Rel. int. (%)	*m/e*	
466, M	9	502, M	36
451	7	484	33
437	–	473	36
393	2	420	27
376	2	403	27
361	6	385	24
333	11	357	24
319	58	346	27
229	17	247	18
220	18	238	18
217	17	235	18
204	14	222	18
191	7	209	18
189	10	204	15
147	33	162	15
129	19	138	9
73	100	82	9

pound is the tetra-TMS derivative of a species possessing a molecular weight of 178 (466 – 288); a likely candidate is a lactone of a hexonic acid. Petersson et al. [18] have reported characteristic intense ions at *m/e* 220 and 319 for the tetra-TMS derivative of glucono-1,5-lactone (II).



II

The spectrum of component D compares very favorably (with the exception of the ion of *m/e* 229) with that of the 1,5-lactone reported by Petersson et al. [18].

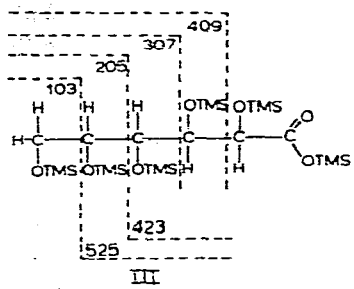
Peaks E and F yielded virtually identical mass spectral data (Table V), indicating isomeric structures. The molecular ion was not detected for either isomer; the M − 15 ion was present but not intense. Loss of trimethylsilanol from *m/e* 525 produced *m/e* 435. Subsequent loss of a second trimethylsilanol produced *m/e* 345. Injection of a sample of trimethylsilyated α- and β-D-glucose produced two major peaks with retention times that were identical to those of peaks E and F (the α-isomer is known to elute prior to the β-isomer [19]). The mass spectra of the unknown and reference standards were identical to those previously reported and described [20].

Compound G (Fig. 2) is tentatively identified as the TMS derivative of mannonic acid. The mass spectral data shown in Table VI compare favorably with those previously published [21]. Unfortunately, the ion of M − 15 (*m/e* 613) was not detected as the spectra were not scanned to >*m/e* 600. The triplet of ions of *m/e* 523, 525, and 538 (TMS) and 565, 570, and 583 (TMS-d_9), however, are strong evidence for this component being a trimethylsilylated hexonic acid. Some of the more important mass spectral peaks can be explained by the fragmentation pattern reported for this compound [21], and shown overleaf (III).

TABLE V

MASS SPECTRAL DATA FOR COMPONENTS E AND F

TMS derivative		TMS-d_9 derivative	Mass shift
m/e	Rel. int. (%)	*m/e*	
(540), M	–	(585), M	45
525	0.2	567	42
435	3	468	33
393	2	426	33
361	2	388	27
345	2	369	24
332	1	359	27
319	2	346	27
305	3	332	27
291	2	315	24
243	2	261	18
231	3	246	15
217	20	235	18
204	100	222	18
191	47	209	18
147	23	162	15
129	7	138	9
117	5	123	6
103	5	112	9
73	66	82	9



III

Ions such as *m/e* 217, 319, 333, 435, and 523 could be formed by loss of trimethylsilanol from *m/e* 307, 409, 423, 525, and 613, respectively. The shift data shown for the TMS-d_9 derivatives in Table VI are consistent with these observations. As Petersson et al. [21] report that the mass spectrum of the derivatized gluconic acid 'differed only slightly from that of mannonic acid', the identification of G as trimethylsilylated mannonic acid must remain tentative.

DISCUSSION

It has been demonstrated that metabolites in spent hemodialysate fluid obtained from an artificial kidney can be identified and determined by combined GC—MS. Six components have been identified or characterized by combined GC—MS. It was initially expected that these compounds would consist primarily

TABLE VI

MASS SPECTRAL DATA FOR COMPOUND G

TMS derivative			TMS-d_9 derivative	Mass shift
m/e	Rel. int. (%)	Rel. int. (%) [21]	*m/e*	
613, M − 15	–	0.5	–	–
538, M − 90	0.2	0.2	583	45
525	0.5	0.5	570	45
523	0.5	0.5	565	42
435	2	3	471	36
433	2	3	466	33
423	5	5	459	36
359	3	4	386	27
333	22	24	360	27
319	12	14	346	27
307	4	5	334	27
305	11	9	332	27
292	21	20	319	27
277	3	1	304	27
217	19	14	235	18
205	18	18	223	18
204	~9	~8		
147	31	27	162	15
103	13	13	112	9
73	100	100	82	9

of acids; however, a number of neutral compounds were detected as well. One of the major components (the peak eluting between E and F, Fig. 2) has thus far not yet been identified; the mass spectral data that were obtained for this peak were inconclusive.

Although combined GC—MS analysis of spent hemodialysate is time-consuming, principally owing to the lengthy method of sample preparation, it provides at present the only way to accomplish the structural studies necessary for conclusive identification of the molecular components associated with uremia. Several of the major components known to be present in hemodialysate, such as uric acid, hippuric acid and xanthine [10] were not observed in this procedure, probably because of the apparent difficulty in derivatizing these compounds with BSTFA on the microgram scale. Efforts are presently underway in these laboratories to utilize GC—MS for the identification of components collected from liquid chromatographic separations of metabolites in used hemodialysate.

ACKNOWLEDGEMENT

This work was supported in part by a grant (CHE 76-08978) from the National Science Foundation. One of the authors (T.L.M.) expresses his grateful appreciation to the Gulf Oil Foundation and to E.I. du Pont de Nemours and Company for a graduate fellowship.

REFERENCES

1 E.C. Horning and M.G. Horning, Methods Med. Res., 12 (1970) 369.
2 E.C. Horning and M.G. Horning, Clin. Chem., 17 (1971) 802.
3 E.C. Horning, M.G. Horning, J. Szafranek, P. van Hout, A.L. German, J.-P. Thenot and C.D. Pfaffenberger, J. Chromatogr., 91 (1974) 367.
4 M.G. Horning, J. Nowlin, C.M. Butler, K. Lertratanangkoon, K. Sommer and R.M. Hill, Clin. Chem., 21 (1975) 1282.
5 J.A. Thompson and S.P. Markey, Anal. Chem., 47 (1975) 1313.
6 B. Dowty, D. Carlisle, J.L. Laseter, and F.M. Gonzalez, Biomed. Mass Spectrom., (1975) 142.
7 F.W. Bultitude, BioMed. Eng., 5 (1970) 240.
8 F.W. Bultitude and S.J. Newham, Clin. Chem., 21 (1975) 1329.
9 G. Perez, A. Rey, M. Micklus and I. Stein, Clin. Chem., 22 (1976) 240.
10 F.C. Senftleber, A.G. Halline, H. Veening and D.A. Dayton, Clin. Chem., 22 (1976) 1522.
11 P.G. McCormick, J. Chem. Educ., 51 (1974) 15.
12 M. Zinbo and W.R. Sherman, Tetrahedron Lett., (1969) 2811.
13 W.C. Butts, Anal. Lett., 3 (1970) 29.
14 W.C. Butts and W.T. Rainey, Jr., Anal. Chem., 43 (1971) 538.
15 J.E. Mrochek and W.T. Rainey, Jr., Anal. Biochem., 57 (1974) 173.
16 C.C. Porter, B.H. Arison, V.F. Gruber, D.C. Titus and W.J.A. VandenHeuvel, Drug Metab. Dispos., 3 (1975) 189.
17 C.E. Dalgliesh, E.C. Horning, M.G. Horning, K.L. Knox and K. Yarger, Biochem. J., 101 (1966) 792.
18 G. Petersson, O. Samuelson, K. Anjou and E. von Sydow, Acta Chem. Scand., 21 (1967) 1251.
19 J.A. Hveding, O. Kjølberg and A. Reine, Acta Chem. Scand., 27 (1973) 1427.
20 D.C. DeJongh, T. Radford, J.D. Hribar, S. Hanessian, M. Bieber, G. Dawson and C.G. Sweeley, J. Amer. Chem. Soc., 91 (1969) 1728.
21 G. Petersson, H. Riedl and O. Samuelson, Sven. Papperstidn., 70 (1967) 371.