926

ORGANIC ACIDS IN HUMAN URINE

Biological and methodological aspects

Joachim Greter

UNIVERSITY OF GOTHENBURG
DEPARTMENT OF CLINICAL CHEMISTRY
SAHLGREN'S HOSPITAL
S-413 45 GOTHENBURG, SWEDEN

ORGANIC ACIDS IN HUMAN URINE

Biological and methodological aspects

AKADEMISK AVHANDLING

som för avläggande av filosofie doktorsexamen
vid Göteborgs Universitet kommer att offentligen försvaras
i Pehr Dubbsalen, Sahlgrenska sjukhuset
fredagen den 4 april 1986
kl 09.15 fm
av

JOACHIM GRETER
Dipl.-Chem.

Avhandlingen baseras på följande arbeten:

I Greter, J., Lindstedt, S., Steen, G. Urinary metabolites of cis-9,10-methylene octadecanoic acid: cis-3,4-methylene hexanedioic acid and related compounds. J Biol Chem 1979; **254**:2807-2813.

II Greter, J., Lindstedt, S., Seeman, H., Steen, G. 3-Hydroxydecanedioic acid and related homologues: urinary metabolites in ketoacidosis. Clin Chem 1980; **26**:261-265.

III Greter, J., Ståhle, G. Septumless injection port for capillary gas chromatography. Anal Chem 1982; **54**:1646-1647.

IV Greter, J., Lindstedt, S., Steen, G. 2,6-Dimethyloctanedioic acid - a metabolite of phytanic acid in Refsum's disease. Clin Chem 1983; **29**:434-437.

V Greter, J., Jacobson C-E. Urinary organic acids: isolation and quantitation for routine metabolic screening. Clin Chem 1986; manuscript.

ABSTRACT

Greter, J., Organic Acids in Human Urine. Biological and Methodological Aspects. Page 1 - 49.
Department of Clinical Chemistry, Gothenburg University, Sahlgren's Hospital, S-413 45 Gothenburg, Sweden. Thesis defended April 4, 1986.

A detailed knowledge of the qualitative and quantitative excretion pattern of organic acids in human urine is essential for the discovery of pathological metabolites of etiological and diagnostic value, especially in the field of inborn errors of metabolism.

The discovery and structure elucidation of three previously unknown urinary metabolites, a new septumless injection port for gas chromatography, and a new isolation method for urinary organic acids are described.

cis-3,4-Methylene hexanedioic acid has been found to be a normal constituent of human urine. The structure was deduced from gas chromatography — mass spectrometry of different derivatives and confirmed by organic synthesis. Both peroral and intraperitoneal administration of labeled and unlabeled cis-9,10-methylene octadecanoic acid to rats led to the excretion of cis-3,4-methylene hexanedioic acid and homologues with six, eight, and nine carbon atoms in the urine.

Patients with ketoacidosis of different etiology excreted up to fifty times more 3-hydroxydecanedioic acid in their urine than healthy individuals. Even saturated and unsaturated homologues of this metabolite with eight, twelve and fourteen carbon atoms were found in the urine of these patients. The structure of the main metabolite was deduced from the mass spectra of different derivatives, and from the fact that dehydration and catalytic hydrogenation yielded decanedioic acid.

Two patients lacking the capability of α-oxidation (Refsum's disease) have been found to excrete 2,6-dimethyloctanedioic acid. Structure elucidation was done by low and high resolution mass spectrometry of different derivatives. The excretion of this metabolite is best explained by the assumption that phytanic acid to a minor extent is degradated by an initial ω-oxidation and subsequent β-oxidation steps.

A new septumless injection port for on-column injection onto capillary columns was developed and tested, both for manual injection and in connection with an automatic sampler. The device has been in use for more than five years in our laboratory.

Poor recovery of polar organic acids from urine with the ether/ethylacetate extraction method prompted us to search for a better isolation method amenable to automation. A column chromatographic isolation method based on Porapak® was therefore developed and tested. This isolation method has in the past four years proven to be very useful in routine analysis of urinary organic acids for metabolic screening. Good correlation was obtained between a mass chromatographic quantitation method for organic acids and the simultaneous quantitation with a flame ionization detector.

Key words: Human urine • Cyclopropane fatty acids • Keto acidosis • Refsum's disease • ω-Oxidation • Urinary organic acids • Metabolic screening • Gas chromatography—mass spectrometry • Septumless injection port • column chromatography

ISBN: 91-7900-012-6. Correspondence to Joachim Greter, Department of Clinical Chemistry, Gothenburg University, Sahlgren's Hospital, S-413 45 Gothenburg, Sweden.

Department of Clinical Chemistry
Gothenburg University
Gothenburg, Sweden

ORGANIC ACIDS IN HUMAN URINE

Biological and methodological aspects

Joachim Greter

Gothenburg 1986

ISBN 91-7900-012-6

To my parents

Ach! was in tiefer Brust uns da entsprungen,
was sich die Lippe schüchtern vorgelallt,
missraten jetzt und jetzt vielleicht gelungen,
verschlingt des wilden Augenblicks Gewalt.
Oft, wenn es erst durch Jahre durchgedrungen,
erscheint es in vollendeter Gestalt.
Was glänzt, ist für den Augenblick geboren;
das Echte bleibt der Nachwelt unverloren.

Goethe JW. Faust, Erster Teil

ABSTRACT

Greter, J., Organic Acids in Human Urine. Biological and Methodological Aspects. Page 1 - 49.
Department of Clinical Chemistry, Gothenburg University, Sahlgren's Hospital, S-413 45 Gothenburg, Sweden. Thesis defended April 4, 1986.

A detailed knowledge of the qualitative and quantitative excretion pattern of organic acids in human urine is essential for the discovery of pathological metabolites of etiological and diagnostic value, especially in the field of inborn errors of metabolism.

The discovery and structure elucidation of three previously unknown urinary metabolites, a new septumless injection port for gas chromatography, and a new isolation method for urinary organic acids are described.

cis-3,4-Methylene hexanedioic acid has been found to be a normal constituent of human urine. The structure was deduced from gas chromatography — mass spectrometry of different derivatives and confirmed by organic synthesis. Both peroral and intraperitoneal administration of labeled and unlabeled cis-9,10-methylene octadecanoic acid to rats led to the excretion of cis-3,4-methylene hexanedioic acid and homologues with six, eight, and nine carbon atoms in the urine.

Patients with ketoacidosis of different etiology excreted up to fifty times more 3-hydroxydecanedioic acid in their urine than healthy individuals. Even saturated and unsaturated homologues of this metabolite with eight, twelve and fourteen carbon atoms were found in the urine of these patients. The structure of the main metabolite was deduced from the mass spectra of different derivatives, and from the fact that dehydration and catalytic hydrogenation yielded decanedioic acid.

Two patients lacking the capability of α-oxidation (Refsum's disease) have been found to excrete 2,6-dimethyloctanedioic acid. Structure elucidation was done by low and high resolution mass spectrometry of different derivatives. The excretion of this metabolite is best explained by the assumption that phytanic acid to a minor extent is degradated by an initial ω-oxidation and subsequent β-oxidation steps.

A new septumless injection port for on-column injection onto capillary columns was developed and tested, both for manual injection and in connection with an automatic sampler. The device has been in use for more than five years in our laboratory.

Poor recovery of polar organic acids from urine with the ether/ethylacetate extraction method prompted us to search for a better isolation method amenable to automation. A column chromatographic isolation method based on Porapak® was therefore developed and tested. This isolation method has in the past four years proven to be very useful in routine analysis of urinary organic acids for metabolic screening. Good correlation was obtained between a mass chromatographic quantitation method for organic acids and the simultaneous quantitation with a flame ionization detector.

Key words: Human urine • Cyclopropane fatty acids • Keto acidosis • Refsum's disease • ω-Oxidation • Urinary organic acids • Metabolic screening • Gas chromatography—mass spectrometry • Septumless injection port • column chromatography

ISBN: 91-7900-012-6. Correspondence to Joachim Greter, Department of Clinical Chemistry, Gothenburg University, Sahlgren's Hospital, S-413 45 Gothenburg, Sweden.

This thesis is based on the following papers:

I Greter, J., Lindstedt, S., Steen, G. Urinary metabolites of cis-9,10-methylene octadecanoic acid: cis-3,4-methylene hexanedioic acid and related compounds. J Biol Chem 1979; **254**:2807-2813.

II Greter, J., Lindstedt, S., Seeman, H., Steen, G. 3-Hydroxydecanedioic acid and related homologues: urinary metabolites in ketoacidosis. Clin Chem 1980; **26**:261-265.

III Greter, J., Ståhle, G. Septumless injection port for capillary gas chromatography. Anal Chem 1982; **54**:1646-1647.

IV Greter, J., Lindstedt, S., Steen, G. 2,6-Dimethyloctanedioic acid - a metabolite of phytanic acid in Refsum's disease. Clin Chem 1983; **29**:434-437.

V Greter, J., Jacobson C-E. Urinary organic acids: isolation and quantitation for routine metabolic screening. Manuscript.

In the following discussion these papers will be referred to by the above Roman numerals.

CONTENTS

1. INTRODUCTION

Mankind has long been fascinated by the vision that, if only all parts of a given system were known, the fate and history of that system would be predictable (1-4).

One such system is the human being himself who for a long time has been the subject of investigations aimed at the complete understanding of all its constituent parts (5).

Because of the bewildering complexity of the human being, investigations have focused on minor subsystems - one of these is the urine, a part of the waste disposal system of the human body (6).

Through the careful investigation of the waste disposal sites of an ancient civilization, an archaeologist may gain insight into how that community once lived (7). The investigation of one of the human bodys waste disposal systems e.g. the urine, may in a similar way give som insight into how the human body works biochemically (8-10).

Although urine is only a part of the human waste disposal system its complexity has forced investigators to focus on different compound classes present in the urine, such as peptides (11,12), amino acids (13), carbohydrates (14), steroids (15), bile acids (16), and organic acids (17), as well as inorganic salts, pigments and nitrogen bases (18). The aims and results of the investigations of all these different compound classes were highly influenced by the available separation and detection methods.

The first records of human urine are those dealing with its antiseptic and healing effects, as well as those claiming that the fate of a human being could in part be foreseen by the investigation of the urine (19,20). Analysis of human urine for diagnostic purposes has certainly been practiced earlier than the first Babylonian written reports (21), but has probably been of the same level of sophistication, where separation at best meant evaporation and detection was equal to visual inspection. It took several thousand years until color reactions, solvent extractions, and crystallisations were used for the identification and quantitation of urinary compounds (8,22). Uric acid, urea, and hippuric acid are among the substances separated and characterized in those years when chemical methods were introduced for the analysis of urinary constituents (8,23). The separation of isolated compounds from impurities was tedious, time

consuming, and required large amounts of substance. The discovery of chromatography at the beginning of this century (24) drastically changed that situation, and impressive advances were made in the elucidation of urinary organic acids with the techniques of paper, thin layer, and column chromatography (17).

After those first steps towards a more specific evaluation of the urinary components for diagnostic and research purposes, it took less than fifty years to arrive at the methods used in this work.

A detailed knowledge of the qualitative and quantitative excretion pattern of organic acids in human urine is essential for the discovery of pathological metabolites of etiological and diagnostic value, especially in the field of inborn errors of metabolism. Knowledge about the structure and metabolism of the urinary constituents has increased in parallel with analytical advances, and such a symbiotic relationship is also reflected in this work, in which the main aim was to gain more knowledge about the acidic compounds of human urine in health and disease (**Papers I, II and IV**). Whilst working with the elucidation of organic acids in human urine, some methodological problems were solved to suit our needs in clinical routine analysis (**Papers III and V**).

2. BIOLOGICAL ASPECTS AND RESULTS

2.1 Metabolism

It has been known for a very long time that some compounds appear to pass the body unchanged, as revealed by the odor or color of the urine after the ingestion of certain foods, whereas others are absorbed and utilized by the body (8,25). Later it was realized that ingested foods where transformed by the body and that even endogenously produced compounds where excreted (8,26). Recently it was recognized that some compounds excreted in the urine originate from the action of the intestinal microflora (27,28).

The first experimental proof, that an ingested compound is transformed by the human body and then excreted into the urine, was made at the beginning of the nineteenth century, when it was shown that ingested benzoic acid to a very great extent could be recovered as hippuric acid from the urine (29-31). Nearly seventy years later it was shown by Knoop, that ingested compounds may not only be conjugated but also degradated

by the body before they are excreted. Knoop was the first to deduce a metabolic pathway from the analysis of urinary metabolites: looking at the excreted degradation products of ω-phenylcarboxylic acids fed to dogs, he derived the β-oxidation sequence for fatty acids (10). In the following decades the β-oxidation pathway of fatty acids in mitochondria was unraveled by numerous workers (32-34), leading to the scheme shown in **Figure 1.**

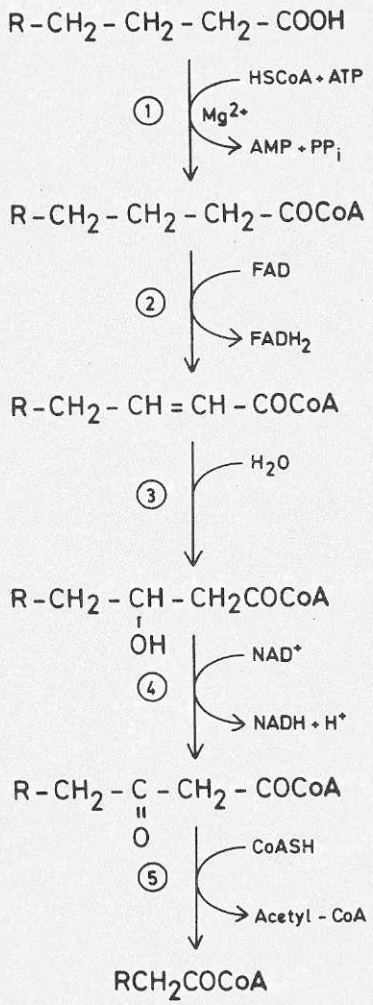

Figure 1. Single β-oxidation cycle with participating enzymes: **1.** Long-chain acylthiokinase (acid:CoA ligase(AMP), EC 6.2.1.2); **2.** Acyl-CoA dehydrogenase (acyl-CoA:oxidoreductase, EC 1.3.2.2); **3.** Enoyl-CoA hydrase (L-3-hydroxyacyl-CoA hydro lyase, EC 4.2.1.17); **4.** β-Hydroxyacyl-CoA dehydrogenase (L-3-hydroxyacyl-CoA:NAD oxidoreductase, EC 1.1.1.35); **5.** β-Ketoacyl-CoA thiolase (acyl-CoA:acetyl-CoA acyl transferase, EC 2.3.1.16).

Thirty years after Knoops discovery it was shown that long chain fatty acids are not only oxidized by β-oxidation but also by ω-oxidation (35), and another three decades later a third oxidative transformation of fatty acids,

α-oxidation, was discovered (36). The main purpose of the oxidative degradation of fatty acids by the β-oxidation pathway is the production of acetyl-CoA (**Fig. 1**), which may be utilized by the citric acid cycle for energy production. Neither ω-nor α-oxidation are able to degradate fatty acids, and one of their functions is to make certain fatty acids amenable to β-oxiation when structures such as methyl branches (37), an acetylenic bond (38), a cyclopropane ring (39), or a 1,2-substituted aromatic ring (unpublished results) block or impair the action of the β-oxidation enzymes. Typical examples for this role of the ω-oxidation system are the oxidative degradation of long chain cyclopropane fatty acids (**Paper I**) and of branched fatty acids (**Paper IV**).

The urine of healthy subjects and patients with different disorders was found to contain cis-3,4-methylene hexanedioic acid. The structure of this compound was elucidated from the mass spectra of different derivatives and by organic synthesis (I).

Rats, given both radioactively labeled and unlabeled cis-9,10-methylene octadecanoic acid perorally, excreted both labeled and unlabeled 3,4-methylene hexanedioic acid and homologues with six, eight, and nine carbon atoms in the urine (I). A schematic drawing of the possible oxidative degradation of cis-9,10-methylene octadecanoic acid is shown in **Figure 2.**

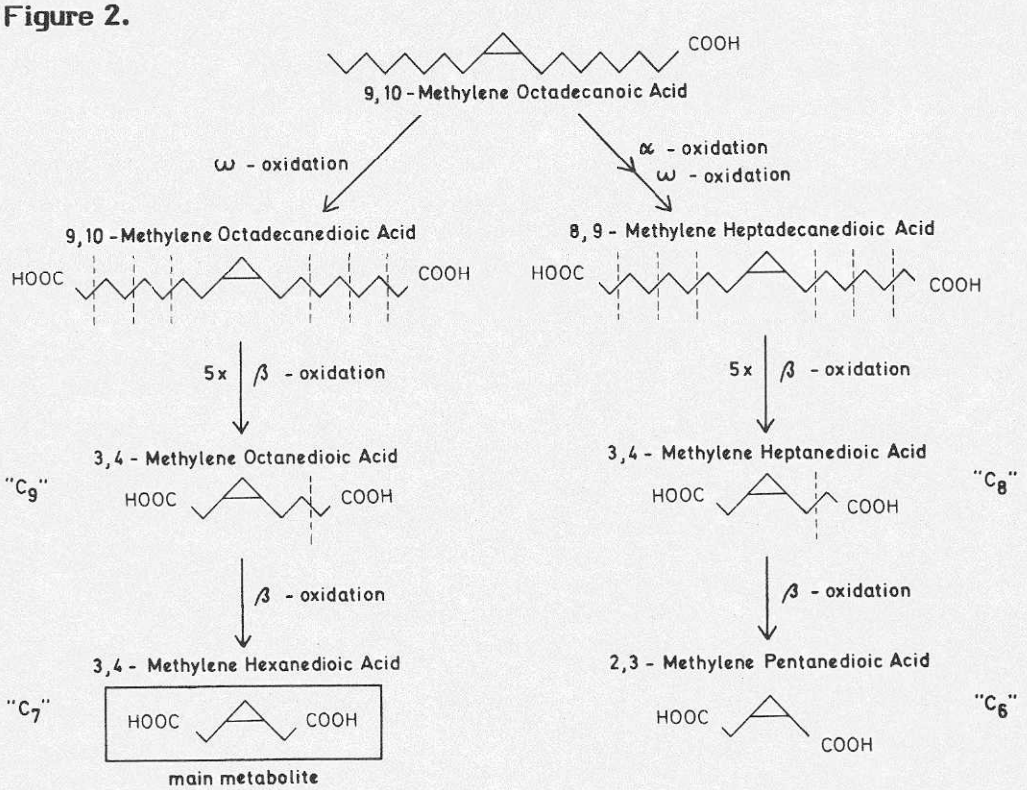

Figure 2. Oxidative degradation of 9,10-methylene octadecanoic acid.

Intraperitoneal administration of cis-9,10-methylene octadecanoic acid to rats led to the excretion of the same metabolites, although at a slower rate than after oral administration. The fact, that both oral and intraperitoneal administration of 9,10-methylene octadecanoic acid yields the same metabolites, supports the view that the short-chain cyclopropane dicarboxylic acids are degradation metabolites from the mammalian organism, and not from gut bacteria (I).

The gastrointestinal uptake of cis-9,10-methylene octanoic acid is comparable to the uptake of stearic acid as shown in **Figure 3** by the recovery of both acids from the lymph of thoracic duct cannulated rats (I).

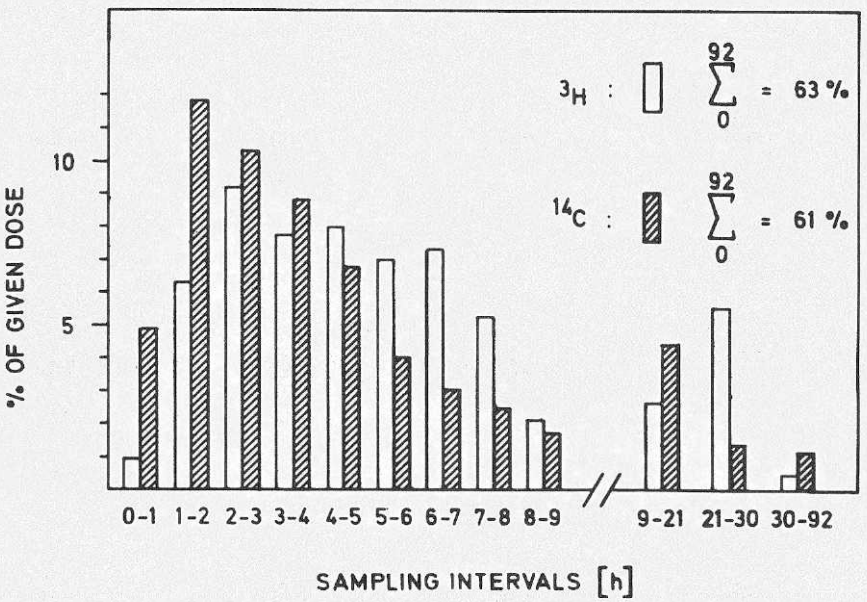

Figure 3. Amount of radioactivity recovered in rat lymph as a per cent of the given amount of [9,10-$^{3}H_2$]cis-9,10-methylene octadecanoic acid methyl ester and [1-^{14}C]stearic acid, respectively.

No metabolic pathway to generate cyclopropane fatty acids has been found in mammals, but long-chain cyclopropane fatty acids are formed in bacteria (40) and certain plants (41). The excretion of cis-3,4-methylene hexanedioic acid found in human urine is therefore most probably due to intestinal absorption of long chain cyclopropane fatty acids.

It is known that a cyclopropane ring incorporated into a long-chain fatty acid can be degradated completely by the oxidative enzymes of microorganisms (42). The large amount of tritiated water found after

administration of [9,10-$^3H_2$]cis-9,10-methylene octadecanoic acid to rats (**Fig. 4**) may indicate the total degradation of the cyclopropane ring but is in contrast to previous findings where no degradation of the cyclopropane ring has been detected in mammals (39,43).

Although about 70% of the ingested cyclopropane fatty acids may be excreted as short-chain carboxylic acids, a minor amount seems to be incorporated into body lipids as shown in **Figure 4** by the radioactivity recovered from the rat carcass (**I**).

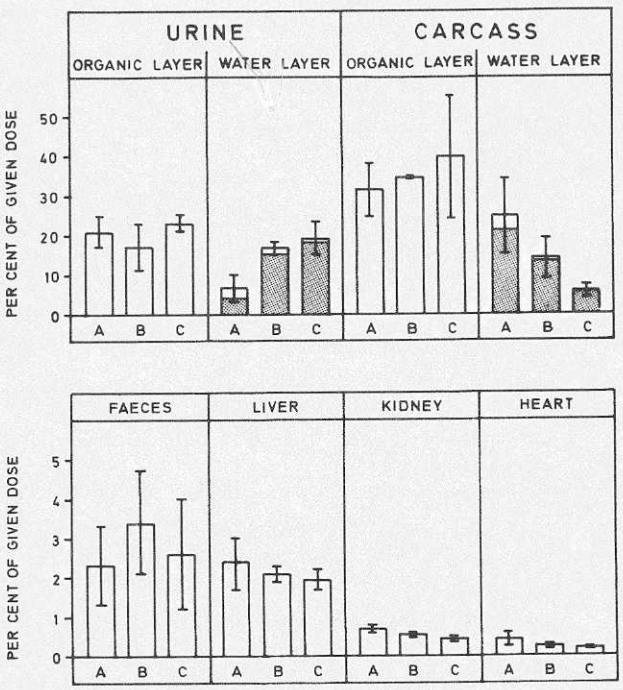

Figure 4. Radioactivity (% of given dose, mean and range of two experiments) from rats fed [9,10-$^3H_2$]cis-9,10-methylene octadecanoic acid methyl ester, and killed after one (**A**), two (**B**), and three (**C**) days.
The shaded areas denote the amount of labeled water found after distillation; the difference is nonvolatile residue.

It has been shown that D-aspartic acid accumulates in brain tissues with age (44). If cyclopropane fatty acids are accumulated in a similar way in different cell membranes during a lifetime, a higher rigidity and stability of those membranes could be expected (45,46), which might contribute to the ageing of some tissues.

The excretion of the metabolite described in **Paper I**, 3,4-methylene hexanedioic acid, does not depend on the state of health. 2,6-Dimethyl-octanedioic acid, the dicarboxylic acid reported in **Paper IV**, on the other

hand is only detected in the urine of patients with a rare metabolic disorder, Refsum's disease.

Phytanic acid, formed from phytol ingested with plant-derived foods (47), can not be oxidized by the β-oxidation system until oxidized at carbon atom two by α-oxidation (48) as shown in **Figure 5.**

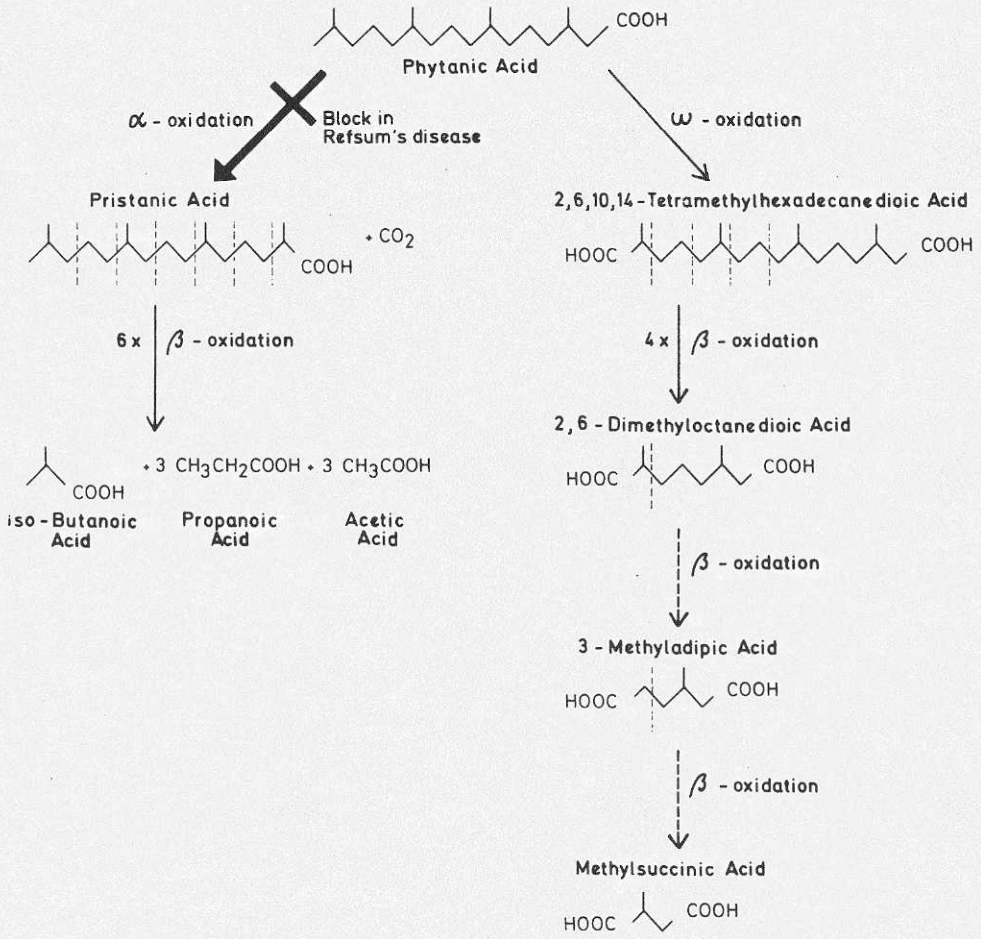

Figure 5. Oxidative degradation of phytanic acid.

In Refsum's disease (49) α-oxidation has been shown to be absent (50), leading to an accumulation of phytanic acid in different tissues. The accumulated phytanic acid impairs the normal neural functions causing symptoms such as blindness, deafness, and loss of movement control in the extremities (49).

As shown by the excretion of 2,6-dimethylocatanedioic acid, phytanic acid can also be made accesible to β-oxidation by an initial ω-oxidation. Unfortunately the capacity of this minor pathway is not sufficient to prevent an accumulation of phytanic acid, if the dietary intake of phytol is not restricted.

In the formation of 3,4-methylene hexanedioic acid (I) and of 2,6-dimethyloctanedioic acid (IV) ω-oxidation becomes important because of structures blocking the β-oxidation system. ω-Oxidation also becomes important when changes of the normal metabolism occur, as in ketogenic conditions. An example for this is the dicarboxylic acid described in **Paper II**, 3-hydroxydecanedioic acid, another dicarboxylic acid normally found in human urine. In healthy subjects only minimal amounts are found, but in ketoacidosis a fifty-fold increase in the excretion may be found, reaching up to 400 µmol/l of urine (II).

It has been shown that endogeneously produced adipic acid - also a normal constituent of urine - is excreted in much higher amounts in ketoacidosis of different etiology (51). The excretion of 3-hydroxydecanedioic acid correlates positively with the excretion of adipic acid in ketoacidosis supporting the view, that 3-hydroxydecanedioic acid is of endogeneous origin. The finding, that even saturated and unsaturated homologues of 3-hydroxydecanedioic acid with eight, twelve, and fourteen carbon atoms can be detected in ketoacidosis (II), is a strong indication for an accumulation caused by an overloading of the β-oxidation system. This shows that in spite of the activation observed for the oxidative degradation of fatty acids in ketotic conditions (52-56), the capacity of the β-oxidation system is not sufficient to cope with the increased availability of long and medium chain fatty acids in ketoacidosis.

2.2 Diagnosis

Already in those times, from which no written records can be found, "physicians" may have noticed urines that have a sweet taste, turn dark on storage, or have a peculiar odor, without knowing the nature or the metabolic origin of the compounds leading to the observed deviations from normal urines. A modern physician would have realized that these patients may suffer from diabetes mellitus, alcaptonuria, or possibly phenylketonuria.

The chemical nature of the compounds leading to these peculiarities of the urine of certain patients have been identified. Thus, in 1815 glucose was identified in the urine of patients with diabetes mellitus (57), in 1891 homogentisic acid was shown to be the compound leading to the darkening of the urine of patients with alkaptonuria (58), and in 1934 phenylpyruvic acid was identified in the urine of patients now known to suffer from phenylketonuria (59).

Among the above mentioned diseases alkaptonuria was one of the disorders for which the term "inborn error of metabolism" was coined by Garrod (60). The concept of the inborn errors of metabolism and the development of the chromatographic methods, especially paper chromatography, lead to the discovery of several errors of amino acids and carbohydrate metabolism (13,14). Later the development of gas chromatography and especially gas chromatography-mass spectrometry and their application to the analysis of organic acids in blood and urine (61) led to the discovery of several inborn errors of metabolism such as isovaleric acidemia (62), methylmalonic aciduria (63), propionic acidemia (64), pyroglutamic aciduria (65), and 3-methylcrotonylglycinuria (66,67).

Metabolic diseases may be detected because of the secondary effects of the underlying genetic disorders, even if the exact genetic location and nature of the defect are not known (68-70). The genetic defect may lead to increased concentrations of metabolites detectable in amniotic fluid, blood, or even urine of the mother, or may be measured as deficient or impaired enzymatic activity in fetal cell cultures (71). Examples of inborn errors detected prenataly in this way are methylmalonic aciduria (72-74), maple syrup urine disease (75,76), propionic acidemia (77,78), 3-hydroxy-3-methylglutaric aciduria (79,80), glutaric aciduria type I (81), and tyrosinemia (82-84).

The pre- and post-natal diagnosis of the above mentioned metabolic disorders leading to abnormal excretion patterns, rely in part on the techniques discussed in the following methodological part of this presentation.

3. METHODOLOGICAL ASPECTS AND RESULTS

3.1 Collection

Already in ancient times physicians knew that the time of collection and storage time of the urine had an effect on the examination results (20). The use of freshly cast morning urine was already advocated in the middle ages as being best for analysis (85), and is still regarded as optimal for analysis, when the total daily amount excreted is not needed for analysis (86).

The analysis should be performed as soon as possible after collection and no preservatives should be added, except when this is required for special analyses. If storage or transport are unavoidable the urine should be kept frozen until analyzed to prevent bacterial growth or chemical decomposition.

3.2 Isolation

The simplest non-selective isolation method for the soluble compounds of the human urine - sediments are not dealt with in this presentation - is the evaporation of urinary water (8). Precipitation (87) may give better selectivity, but the most selective isolation methods are all based on the partition equilibria of the dissolved urinary compounds with a solid, liquid, or gaseous phase.

Purging the urine with an inert gas and subsequent trapping of the compounds carried over is still the method of choice for the analysis of volatile compounds (88-90), although a method for the direct separation and detection of volatiles in aqeous samples has been published (91).

The oldest method for the isolation of the non-volatile urinary organic acids, based on liquid-liquid partition equilibria (92), is still the most widely used because of its simplicity and speed (93,94). Methods depending on liquid-solid partition equilibria have traditionally been based on oxidized silica or alumina to adsorb the non-volatile solutes (95-98). Now a large number of workers use ionic and non-ionic organic polymeric materials to isolate organic acids from human urine (93,94).

When our laboratory started analyzing urinary organic acids for metabolic screening in late 1974, the solvent extraction method was adopted. Because of the known limitations of the solvent extraction method to recover very polar acidic compounds (99,100) we started to search for a better isolation method amenable to automation. After a lot of disappointing experiments with different organic materials we started to evaluate the different Porapak (Trademark of Waters Ass.) materials, and developed the isolation procedure presented in **Paper V**, which is based on a 1:1 by volume mixture of Porapak T and Porapak Q. Acidified samples are applied to the acid-washed columns and eluted with acetonitrile. Good recoveries for unpolar and polar compounds are obtained as shown in **Table 1.**

Table 1. Recovery of selected organic acids as a percent of initial radioactivity (mean ± SD)

	Porapak n=10	Solvent extraction n=10	Ion exchange n=3
Oxalic acid	30.6 ± 5.8	31.3 ± 1.4	94.2 ± 2.0
Lactic acid	34.1 ± 5.4	22.6 ± 0.9	81.1 ± 2.4
Succinic acid	93.6 ± 2.2	74.0 ± 3.2	94.4 ± 1.8
2-Oxoglutaric acid	97.0 ± 1.2	58.4 ± 2.5	83.9 ± 2.2
Citric acid	66.3 ± 4.5	8.7 ± 0.5	85.5 ± 1.1
Cinnamic acid	94.5 ± 3.1	95.4 ± 3.6	99.2 ± 3.8

The main advantages of the isolation of acidic compounds from urine with the method based on Porapak material over the solvent extraction method with ethyl acetate is illustrated in **Figure 6.** The most prominent features are the reduced recovery of urea and phosphoric acid (peaks 4 and 5 in **Figure 6**), and the increased recovery of polar compounds e.g. citric acid (peak 12 in **Figure 6**).

The Porapak columns are regenerated by washing with acetone and water, and may be reused more than fifty times. Deterioration of the material is shown by a decreased recovery of such polar compound as the tetronic acids. The column material is routinely replaced once each month in our laboratory.

The adsorption mechanism on the Porapak material has not been studied in detail but the most likely explanation is a combination of hydrophobic forces of the ethylvinylbenzene-dimethylbenzene co-polymer with hydrophilic forces of the enclosed water molecules.

Even compounds other than organic acids are recovered from the Porapak columns such as glycerol (101), 3-methylcrotonylglycine (102), tyrosine (**V**), and orotic acid (**V**).

Simple separations through gradient elution can also be achieved on these materials, but that possibility has not been investigated in detail.

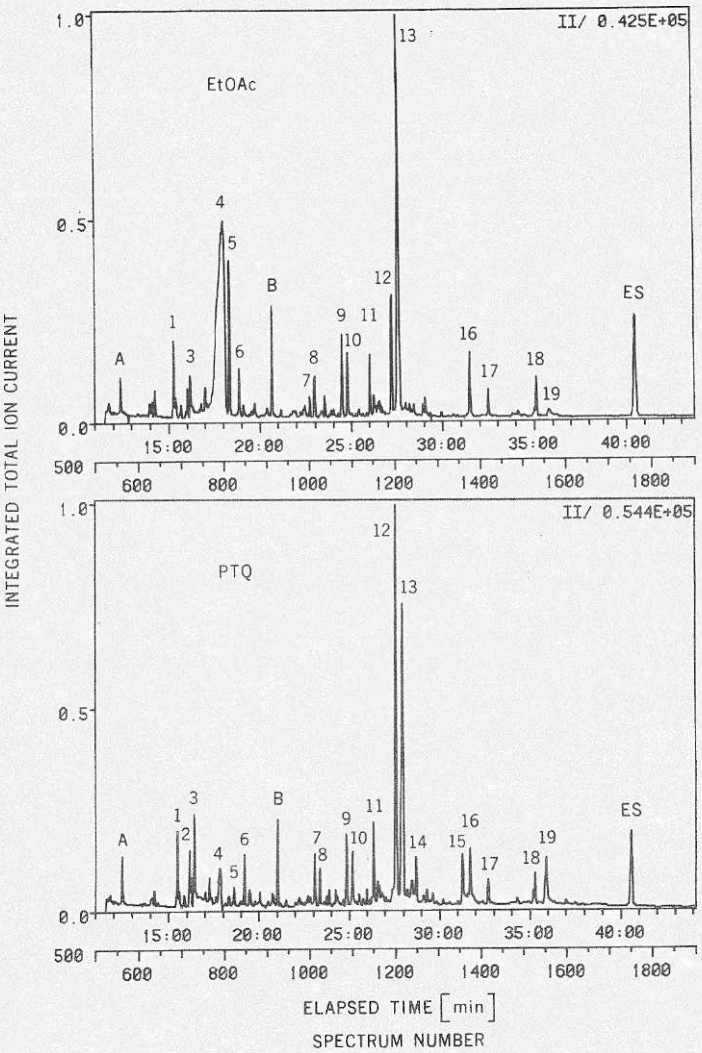

Figure 6. Total ion current traces of methoxymated and silylated compounds isolated from 2 ml of pooled normal urine with ethyl acetate extraction (EtOAc, upper trace) and with adsorption on Porapak^{R} (PTQ, lower trace). Prominent peaks are: **1** procedural artifact; **2** p-cresole; **3** sulfuric acid; **4** urea; **5** phosphoric acid; **6** succinic acid; **7** pyroglutamic acid; **8** furane-5-hydroxymethyl-2-carboxylic acid; **9** 4-hydroxyphenylacetic acid; **10** glycylfurane-2-carboxylic acid; **11** aconitic acid; **12** citric acid; **13** hippuric acid; **14** unidentified hexose; **15** uric acid; **16** 3-hydroxyhippuric acid; **17** 4-hydroxyhippuric acid; **18** unidentified hexonic acid; **19** N-phenylacetylglutamine; **A** 4-pentynoic acid (internal standard); **B** 3-chlorobenzoic acid (internal standard); **ES** tetracosanoic acid (external standard).

Chromatographic conditions: 23 cm/s He carrier gas; 20 m, 0.5 mm I.D. pyrex-glass capillary column coated with 1.2 µm of cross linked SE-54; temperature programmed from 50°C, held for 2 min, up to 290°C, held for 9 min, at a rate of 7°C/min.

3.3 Separation

The early attempts to separate the soluble urinary compounds isolated were made by distillation, consecutive selective solvent extractions, or recristallisations after proper chemical modifications (8,31). A still ongoing revolutionary development was started with the first papers on column chromatographic separation (24,103-106). Other milestones were the development of paper chromatography (107), thin layer chromatography (108-110), and especially gas chromatography (111-115).

Although most of the urinary organic acids may be separated in their native form by liquid chromatography (116,117), it is often advantageous to modify them chemically before chromatography is started. The chemically modified organic acids may give a better detection sensitivity, or may have better chromatographic properties. Most organic acids must be derivatized to increase their volatility and stability for gas chromatographic separation, which has become the accepted standard method for the analysis of urinary organic acids, first with packed columns (118,119), and then with capillary columns (93,94,120).

A schematic illustration of a gas chromatograph (GC) is shown in **Figure 7** (detail G), and consists of a pressurized carrier-gas delivery system, a sample application port, a chromatographic column, and a detector. The electric signal from the detector is amplified and transferred to a recorder or data system.

Capillary columns may be prepared from metals, plastics, different glasses, and from pure fused silica (121). Different ways to apply the chromatographic phase onto the unmodified or modified inner wall of the capillary column have been described (122). The most common is now the static coating method where a solution of the chromatographic phase is introduced into the capillary column. After the subsequent evaporation of the solvent the chromatographic phase remains as a thin homogeneous coating on the inner wall of the capillary column (123). It is essential to make the coating non-extractable for on-column injections, and several methods to stabilize the chromatographic phase by crosslinking have been published (124-129). A careful selection of the material and internal diameter of the capillary column as well as the thickness and nature of the coating is crucial for each application (121,122).

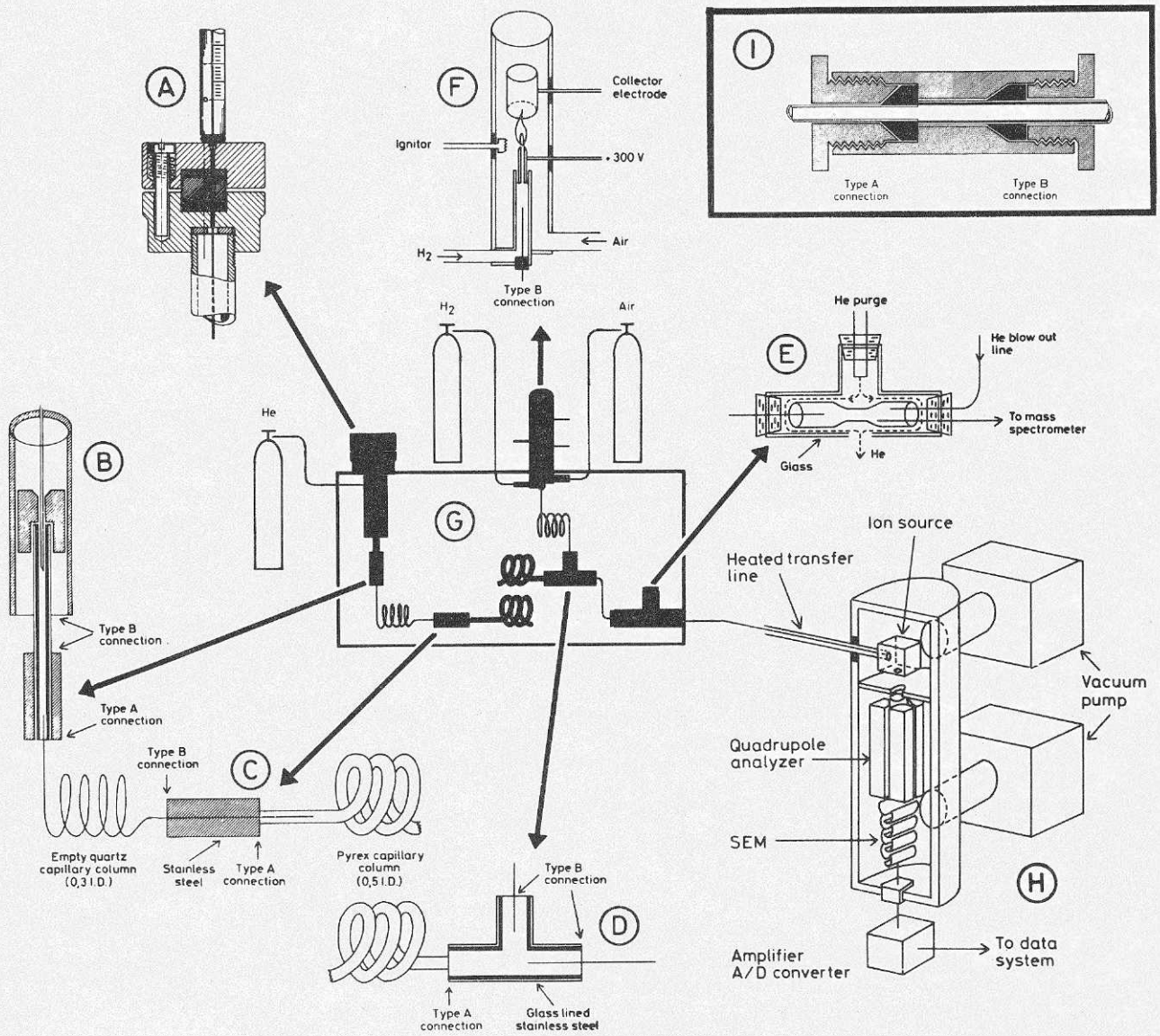

Figure 7. Schematic drawing of a combined gas chromatograph - mass spectrometer. **A:** septumless injection port; **B:** "quasi on column" glass-lined stainless-steel injector; **C:** coupling of empty fused silica precolumn (0.3 mm I.D.) to analytical wide bore (0.5 mm I.D.) pyrex glass column; **D:** glass-lined stainless steel effluent splitter; **E:** open-split coupling; **F:** flame ionization detector; **G:** gas chromatograph; **H:** quadrupole mass spectrometer; **I:** detail of the dead volume free connection used.

Because of their higher sample capacity wide-bore capillary columns with a thick-film coating appeared to be the best choice for us to use in the routine analysis of organic acids in urine (130,131). When we started to analyze organic acids in urine, appropriate wide-bore capillary columns were not available commercially, and were therefore prepared in our

laboratory. Although different cross-linked thick-film wide-bore capillary columns are now commercially available, we still prefer to prepare the columns in our laboratory because of economic and didactic considerations (132).

The step from packed columns to wide-bore (0.5 mm I.D.) capillary columns illustrated in **Figure 8** was done in 1978 in our laboratory and led to the development of the septumless injection port described in **Paper III.** This injection port eliminated all the problems we previously had with the injection membrane, especially bleeding and particles torn from the membrane which were flushed into the capillary column, leading to a severe impairment of the chromatographic performance.

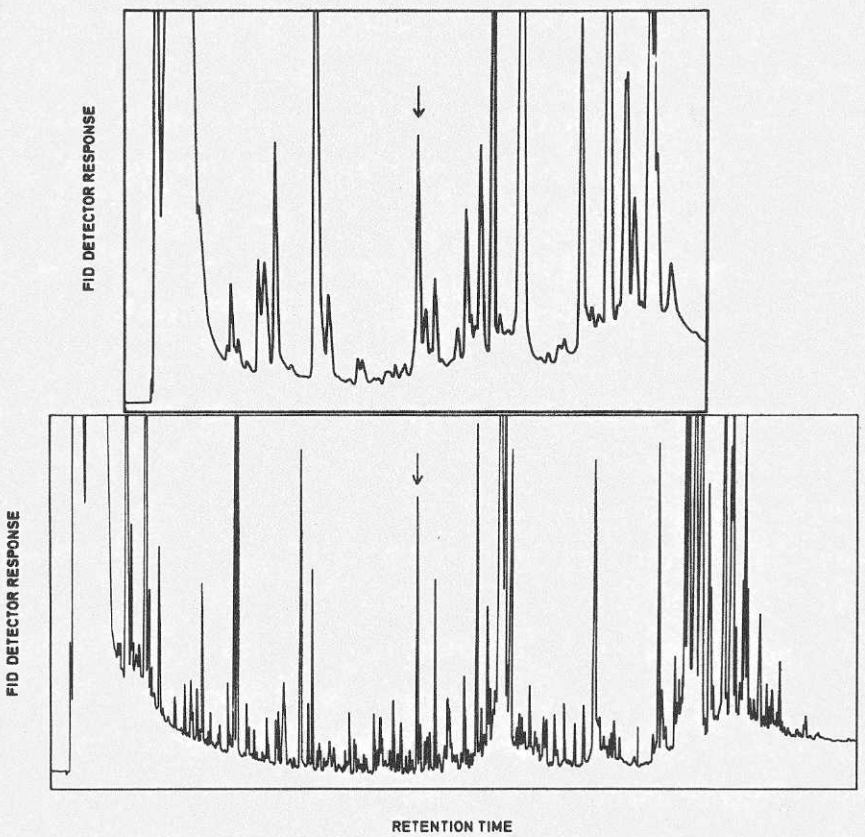

Figure 8. Comparison of packed (upper) and capillary (lower) column chromatogram of a patients urine after isolation, methoxymation, and silylation. The arrow indicates the position of the internal standard.

The injection port (**Fig. 7,** detail A) was interfaced to a modified autosampler and fitted with a wide bore (0.7 mm I.D.) glass-lined stainless-steel tubing to enable "quasi on column" injection (**Fig. 7,** detail B). This set

up facilitates the connection of all sorts of capillary columns to a given injector configuration thus eliminating the need for special on column injection devices. The shown set up has been in use in our laboratory for manual injection since 1978 and for autosampler injection since 1981 for both flash heated as well as cold "quasi on column" injections.

3.4 Detection

Before the advent of chromatographic separation techniques the detection of urinary constituents has since ancient times relied on the inspection of the urines color, transparency, odor, and taste (133,134).

The introduction of the chromatographic techniques made it possible to separate a large number of urinary compounds, most of them colorless. The inability to detect the separated compounds while they eluted from the chromatographic column catalyzed the development of different detectors. Some of the detectors now in use in liquid column chromatography are the refractive index detector (135), the UV-detector (136-138), the fluorescense detector (139), and the electrochemical detector (140,141).

Two gas chromatographic detectors of historical interest are Martin's gas density balance, and Cook's argon detector (142). Now the most widely used detector in gas chromatography has become the flame ionization detector (113,143-146). Other detectors used in gas chromatography are the thermal conductivity detector (147,148), the electron capture detector (149), the photoionization detector (150), and the infrared spectrometer (151-153).

The UV-detector, the fluorescense detector, the electro-chemical detector, the electron-capture detector, the photoionization detector, and the infrared spectrometer can be operated to specifically detect certain compounds or compound classes. All other detectors mentioned have no special compound specificity, and are thus useful for the detection of nearly all urinary organic compounds.

The most versatile detector so far developed suitable for eluents in liquid and gas chromatography is the mass spectrometer (154-162). The mass spectrometer is unique because it combines high sensitivity with the ability to generate very specific spectra for nearly all compounds, either in their native state or as suitable derivatives (163-172). One disadvantage of

the mass-spectrometric detector shared by the flame ionization detector is that it is a destructive detector, and thus a compound detected with a mass spectrometer or a flame ionization detector can not be recovered as it was introduced.

For the analysis of urinary organic acids we have chosen to evaluate the effluent of the gas chromatographic column with two independent detectors simultaneously by splitting the effluent through a glass-lined stainless-steel T-piece to a flame ionization detector and to a mass spectrometer. Details of the effluent splitter and of the slightly modified open-split coupling to the mass spectrometer are shown in **Figure 7** (details **D** and **E**, respectively).

The flame ionization detector (FID) consists of a hydrogen flame into which the effluent from the gas-chromatographic column is directed for combustion at a temperature between 1000 and 2000^{o}C (173). At this flame temperature ions are formed, which can be detected by a cylindrical electrode surrounding the flame, and held at a potential of approximately 300 V against ground (**Figure 7**, detail **F**). The current detected is amplified and recorded. Stable operating conditions are obtained for each detector design at certain flow rates of hydrogen gas, air and carrier gas for a given temperature which should be high enough to prevent condensation of material eluting from the chromatographic column.

A dynamic range of six to eight orders of magnitude may be attained depending on the detected compound (174). The lowest detectable amount for favorable detection conditions is in the order of 10^{-12} g/sec (174). This means that a given amount of eluate will yield a higher detector response when passing the detector in a shorter period of time, as long as the time constants of the amplifying and recording systems are sufficiently short. This differential property of the flame ionization detector is shared by the mass-spectometric detector which has about the same sensitivity and dynamic range of detection (175).

A mass spectrometer (MS) is a device that generates volatile ions and separates them in vacuo according to their mass to charge ratio. A schematic diagram of a quadrupole mass spectrometer is shown in **Figure 7** (detail **H**). Ions produced in the source are separated in the mass analyzer section and accelerated against the conversion dynode of a secondary electron multiplier (SEM). The amplified signal from each ion hitting the

conversion dynode is transmitted to a galvanometric recorder or to a data system. Several mass spectrometric analyzers are available, but only the quadrupole and the magnetic sector analyzer in combination with a gas chromatograph are widely used for the analysis of organic acids in urine (93,94,176).

In the ion source both positive and negative ions are formed, usually in favor of the positive ions by a factor of a thousand (156). For some electron-capturing compounds or derivatives the proportion of negative ions may increase considerably and their detection instead of the positive ions can be advantageous (177,178). Ions may be generated in several ways. The most common way is still the electron impact ionization with thermal electrons from a metal filament (often Rh). The thermal electrons are usually accelerated to an energy of about 70 eV. Additional ionization methods used are chemical ionization (179,180), fast atom bombardment (181,182), field ionization and field desorption (183,184), atmospheric pressure ionization (185), and thermo-spray ionization (186,187). Only electron impact and chemical ionization methods are commonly used in the gas chromatographic-mass spectrometric analysis of urinary organic acids.

The combination of gas chromatography with mass spectrometry (GC-MS) generates some specific problems (157,175). Gas chromatography is a high pressure technique (about atmospheric pressure at the column outlet), whereas mass spectrometry depends on a very low pressure (about 10^{-8} torr in the analyzer section). Differential pumping systems have solved that problem. Another mismatch was, that the gas flow rate of a packed gas chromatographic column is in the order of 30 ml/min, whereas the mass spectrometer is able to handle a flow rate in the order of 3 ml/min. Early solutions to that problem were gas separators (157), which have become unnecessary after the advent of capillary columns commonly operating at flow rates below 3 ml/min. Direct introduction of the effluent of the gas chromatographic capillary column into the ion source of the mass spectrometer is thus possible, but has the disadvantage that even solvents and reagents applied with the sample onto the column are pumped into the mass spectrometer. Column effluent diverting systems, such as the open-split coupling shown in **Figure 7** (detail E), are therefore used in many laboratories (188-191).

Another problem is that high performance capillary columns are able to resolve about ten components per second, whereas modern mass spectrometers can at present only sample three full spectra (from m/z 50 to 800) of good quality per second. The chromatographic separation must thus be run below optimal speed which leads to a lower detectability because of sample adsorption and degradation, as well as because of the differential nature of the mass-spectrometric detector (192). This problem is not encountered when the mass spectrometer is operated to detect only certain ions instead of full mass spectra.

3.5 Identification

In the same way as detection of urinary compounds has long relied on specific colors, odors, or tastes for unseparated as well as separated compounds, so has identification (8).

The development of specific separation techniques such as precipitation, distillation, recrystallisation, and especially chromatography enabled the purification of individual urinary components. Once purified a compound could be identified by its physical and chemical properties, such as melting point, boiling point, chemical reactivity towards different reagents, ability to give crystalline products, spectroscopic properties, elemental analysis, and finally by its identity with a synthesized compound showing the same chemical and physical properties.

Although the combined application of physical methods such as infrared spectrometry (IR), nuclear magnetic resonance (NMR), and mass spectrometry (MS) are often sufficient to characterize a compound, the synthetic proof is still the most definitive (193,194).

In the field of urinary organic acid analysis, capillary gas chromatography-mass spectrometry has become the method of choice for fast identification of isolated compounds, simultaneously to the chromatographic separation (195). Liquid chromatography-mass spectrometry might become an alternative to gas chromatography - mass spectrometry because of some recent developments made in the coupling of liquid chromatography with mass spectrometry (196-198). Using the techniques of low and high resolution gas chromatography-mass spectrometry the knowledge about urinary organic acids has increased considerably during the last ten years through the efforts of numerous workers (93,94,199-210).

Because of the large amount of analytical data generated by a gas chromatograph - mass spectrometer the combination with a data system has been essential for data reduction and evaluation (157,175,211-220). Nevertheless many of the early results were obtained without the help of a data system. Datorized gas chromatographic-mass spectrometric equipment is now commercially available from a number of vendors with different types of mass analyzers and with a high degree of reliability and versatility.

Although sophisticated datorized gas chromatographic-mass spectrometric equipment has revolutionized the identification of e.g. organic acids in urine, the analyst will always be needed because of problems with unresolved eluents (221), impurities (222,223) and procedural artifacts (224-226).

3.6 Quantitation

Qualitative data are often sufficient for the correct diagnosis of a disease, but when comparisons, evaluation of severity, and prediction of outcome are to be made quantitative data are indispensable. Quantitative methods introduced very early were gravimetry (8,31), titration (227,228) and colorimetry (229) which are still in use parallel to the newer physical methods. All the detectors mentioned in the previous section are useful for quantitative estimations.

The flame ionization detector and the mass spectrometer have gained worldwide acceptance for the quantitative estimation of urinary organic acids, and are also used in our laboratory. The following discussion will therefore be restricted to the methods of gas chromatography-flame ionization detection and gas chromatography-mass spectrometry.

The mass spectrometric detector may be operated either in the full scan mode or in the single or multiple ion detection mode, usually computer controlled. Higher sensitivity is obtained in the latter operating mode which is thus used for qualitative and quantitative trace analysis (230-233). The full scan mode is used for the structure elucidation of unknown compounds but can be used for quantitative analysis as well, although with lower precision and sensitivity (234,235).

The reliability of quantitative results depends more or less on several factors such as care in the preparation of samples and standards, chroma-

tographic conditions, type, condition and operating mode of the detector, mode of data evaluation, as well as skills and experience of the analyst. Following published recommendations for good laboratory practice (235-239) will prevent the most serious errors in sample and standard handling procedures. The chromatographic conditions must be optimized for each analytical problem by the proper choice of carrier gas, injection method, type of column, type of chromatographic phase, temperature of separation, as well as type and temperature of the connection to the detector. Following the manufacturers installation, test and maintenance procedures for the chosen detector will in most cases be sufficient to obtain satisfactory quantitative results.

Quantitative results from mass spectrometers are highly affected by improper threshold settings, which in addition may be unstable and thus make day to day comparisons difficult. New standard curves are therefore necessary for each measuring sequence (232). Other problems when attempting quantitation of complex biological samples may arise from non linearity of standard curves (240,241), isotope effects (242), choice of internal standard (243,244), as well as instrumental set up and measuring conditions (245).

Even a correct quantitation may be difficult to interpret because of day to day variations in excretion (246-250), excretional changes due to medication (251) or diet (252), and difficulties with interpatient comparisons mainly because of creatinine excretion variances (253-260).

4. CONCLUDING REMARKS

A detailed knowledge of normal metabolic pathways and the variation of normal and pathological urinary excretion patterns is essential for the correct diagnosis and treatment of many inborn errors of metabolism.

Several computer programs for the automatic identification and quantitation of gas chromatographic-mass spectrometric data have been developed (199,212,217). Even programs for the automatic diagnosis of diseases based on gas chromatographic-mass spectrometric data have been published (261). Unfortunately these data programs have not to a significant extent found their way into routine clinical laboratories and seem to work best in the hands of those who developed them.

Other analytical improvements such as sophisticated combinations of different analytical techniques (262), e.g. gas chromatography-fourier transform infrared spectrometry-mass spectrometry-data system (263), which have a great potential for the elucidation of unknown compounds and can with great reliability be used to identify known ones, are to expensive or to complicated for routine clinical laboratories.

It seems therefore unlikely that the "manual" evaluation of analytical data for the purpose of identification and diagnosis of inborn errors of metabolism will be replaced by fully automated computerized methods in the near future.

6. ACKNOWLEDGEMENTS

I express my sincere gratitude to

Professor Sven Lindstedt for his advice and support throughout this work and for his understanding attitude during those times when private life complications impaired the progress of this thesis.

Docent Göran Steen for rewarding collaboration and valuable discussions during the early part of this work.

Birgitta Eliasson for patient and skillful technical assistance in the laboratory as well as for much help as a friend.

Ammie Fredholm as well as Anna-Greta Janson for their indefatigable deciphering and transformation of my hieroglyphic annotations into readable manuscripts, and for much help with funds, employment, technicians and other worldly things during the last eleven years.

Carl-Eric Jacobson for stimulating collaboration and for relieving me from nearly all routine duties during the last part of this work.

Göran Ståhle, Nils Ekendahl and Lennie Svensson for expert and friendly technical assistance.

Sten-Eric Lindgren for most of the nearly perfect drawings, figures and photographs.

Ann McKinnon for patient secretarial help.

The staff of the biomedical library for the numerous references retrieved and copied for me during the years.

All those who have been kind and helpful since the beginning of my stay here in Sweden.

I am especially indebted to

My daughter Tanja for being such a lovely and good girl in spite of all the hard times endured.

My wife Frauke for her love, encouragement and support which made the completion of this work possible.

This work was supported by grants from the Swedish Medical Research Council (13X-585), the Ragnar Söderbergs Stiftelse, the Swedish Council for Planning and Coordination of Research (80/1385), the Fund for Nongraduated Students at the Medical Faculty of the Gothenburg University, and the Sahlgren's Hospital Funds.

7. REFERENCES

1. Schuster P. Zwischenmolekulare Kräfte - Ein Beispiel für das Zusammenwirken von Theorie und Experiment. Angew Chem **93**, 532-553 (1981).

2. Prigogine I. Zeit, Struktur und Fluktuationen (Nobel-Vortrag). Angew Chem **90**, 704-715 (1978).

3. Eigen M. Darwin und die Molekularbiologie. Angew Chem **93**, 221-229 (1981).

4. Berg P. Zerlegung und Rekonstruktion von Genen und Chromosomen (Nobel-Vortrag). Angew Chem **93**, 885-893 (1981).

5. Neuburger M. Geschichte der Medizin I. F Enke, Stuttgart (1906).

6. Montgomery R, Dryer RL, Conway TW, Spector AA. Biochemistry - A case-oriented approach. 3rd ed., C.V. Mosby Comp., St. Louis (1980) p 2.

7. de Laet SJ. Arkeologins Metoder och Problem. (Orig.title:L'archéologie et ses problèmes). Natur och Kultur, Stockholm (1957).

8. Berzelius JJ. Föreläsningar i Djurkemien II. Marquard, Stockholm (1808).

9. Garrison FH. History of Medicine. 4th ed., Saunders Comp., Philadelphia (1929) p 474.

10. Knoop F. Der Abbau aromatischer Fettsäuren im Tierkörper. Beitr Chem Physiol Path **6**, 150-162 (1904).

11. Skarżyński B, Sarnecka-Keller M. Peptides in human urine. Adv Clin Chem **5**, 107-134 (1962).

12. Pruzanski W, Ogryzlo MA. Abnormal proteinuria in malignant diseases. Adv Clin Chem **13**, 335-382 (1970).

13. Bigwood EJ, Crokaert R, Schram E, Soupart P, Vis H. Amino Aciduria. Adv Clin Chem **2**, 201-265 (1959).

14. Sidbury Jr JB. The nonglucose melliturias. Adv Clin Chem **4**, 29-52 (1961).

15. Bush IE. Determination of estrogens, androgens, progesterone, and related steroids in human plasma and urine. Adv Clin Chem **12**, 57-139 (1969).

16. Street JM, Trafford DJH, Makin HLJ. The quantitative estimation of bile acids and their conjugates in human biological fluids. J Lipid Res **24**, 491-511 (1983).

17. Nordmann J, Nordmann R. Organic acids in blood and urine. Adv Clin Chem **4**, 53-120 (1961).

18. Gershenfeld L. Urine and Urinalysis. 3rd ed., Romaine Pierson Publishers, New York (1948).

19. Brockhaus Enzyklopädie **8**, 17th ed., Wiesbaden (1969) p 184.

20. Plinii Secundi C. Historiae Naturalis **XXVIII**: 18-19. Caroli Tauchnitii. Lipsiae (1830).

21. Fåhraeus R. Läkekonstens Historia I. Bonniers, Stockholm (1944) p 78.

22. Neubauer und Vogel. Anleitung zur Qualitativen und Quantitativen Analyse des Harns. Analytischer Theil. 10th ed., 3rd printing, H Huppert, Ed., Kreidel's Verlag, Wiesbaden (1898).

23. Fieser LF, Fieser M. Organische Chemie. Verlag Chemie, Weinheim/Bergstrasse (1968) p 2.

24. Tswett M. Physikalisch-chemische Studien über das Chlorophyll. Die Adsorptionen. Ber deut botan Ges **24**, 316-323 (1906).

25. Hippocrates. Regimen **II**:XXXIX-LX. WHS Jones, Ed., W Heinemann, London (1923).

26. Fruton JS. Biochemistry and Clinical Chemistry - A Retrospect. J Clin Chem Clin Biochem **20**, 243-252 (1982).

27. Bakke JE, Gustafsson J-Å, Gustafsson BE. Metabolism of propachlor by the germfree rat. Science **210**, 433-435 (1980).

28. Bakke J, Struble C, Gustafsson J-Å, Gustafsson B. Catabolism of premercapturic acid pathway metabolites of naphthalene to naphthols and methylthio-containing metabolites in rats. Proc Natl Acad Sci USA **82**, 668-671 (1985).

29. Schmidt JE. Medical Discoveries. CC Thomas, Springfield (1959) p 216.

30. Wöhler F. Ueber die im lebenden Organismus vor sich gehende Umwandlung der Benzoësäure in Hippursäure. Annalen Phys Chem **1842**, XVI:638-641 (1842).

31. Liebig J. Die Organische Chemie in ihrer Anwendung auf Physiologie und Pathologie. Vieweg, Braunschweig (1842).

32. Green DE, Allmann DW. Fatty acid oxidation. In: Metabolic Pathways. 3rd ed., Academic Press, New York (1968) pp 1-36.

33. Stumpf PK. Metabolism of fatty acids. Ann Rev Biochem **38**, 159-212 (1969).

34. Wakil SJ. Lipid Metabolism. Academic Press, New York (1970) pp 1-77.

35. Verkade PE, Elzas M, van der Lee J, de Wolff HH, Verkade-Sandbergen A, van der Sande D. Untersuchungen über den Fettstoffwechsel. I. Hoppe Seyler's Z Physiol Chem **215**, 225-257 (1933).

36. Levis GM, Mead JF. An α-hydroxy acid decarboxylase in brain microsomes. J Biol Chem **239**, 77-80 (1964).

37. Bergström S, Borgström B, Tryding N, Westöö G. Intestinal absorption and metabolism of 2:2-dimethylstearic acid in the rat. Biochem J **58**, 604-608 (1954).

38. von Wagner H, Ritzel G, Bernhard K. Das biologische Verhalten von Fettsäuren mit Dreifachbindung. I. Speicherung und Abbau von Stearolsäure bei der Ratte. Helv Chim Acta **49**, 436-440 (1966).

39. Wood R, Reiser R. Cyclopropane fatty acid metabolism: physical and chemical identification of propane ring metabolic products in the adipose tissue. J Am Oil Chem Soc **42**, 315-320 (1965).

40. Kaneshiro T, Marr AG. Cis-9,10-methylene hexadecanoic acid from the phospholipids of Escherichia coli. J Biol Chem **236**, 2615-2619 (1961).

41. Christie WW. Cyclopropane and cyclopropene fatty acids. In: Topics in Lipid Chemistry I. FD Gunstone, Ed., Logos Press, London (1970) pp 1-49.

42. Tipton CL, Al-Shathir NM. The metabolism of cyclopropane fatty acids by Tetrahymena pyriformis. J Biol Chem **249**, 886-889 (1974).

43. Chung AE. Metabolism of cyclopropane fatty acids. Oxidation of cis-9,10-methylene hexadecanoic and cis-9,10-methylene octadecanoic acids by rat-liver mitochondria. Biochim Biophys Acta **116**, 205-213 (1966).

44. Man EH, Sandhouse ME, Burg J, Fisher GH. Accumulation of D-aspartic acid with age in the human brain. Science **220**, 1407-1408 (1983).

45. Carroll KK. Dietary fat and the fatty acid composition of tissue lipids. J Am Oil Chem Soc **42**, 516-528 (1965).

46. Turner DL, Silver MJ, Baczynski E, Holburn RR, Herb SF, Luddy FE. The synthesis of phosphatidylethanolamine and phosphatidylserine containing acetylenic or cyclopropane fatty acids and the activity of these phosphatides in blood coagulation. Lipids **5**, 650-657 (1970).

47. Steinberg D, Avigan J, Mize C, Eldjarn L, Try K, Refsum S. Conversion of [U-C^{14}]-phytol to phytanic acid and its oxidation in heredopathia atactica polyneuritiformis. Biochem Biophys Res Comm **19**, 783-789 (1965).

48. Avigan J, Steinberg D, Gutman A, Mize CE, Milne GWA. Alpha-decarboxylation, an important pathway for degradation of phytanic acid in animals. Biochem Biophys Res Comm **24**, 838-844 (1966).

49. Steinberg D. Phytanic acid storage disease (Refsum's disease). In: The Metabolic Basis of Inherited Disease. 5th ed., JB Stanbury, JB Wyngaarden, DS Fredrickson, JL Goldstein, MS Brown, Eds., McGraw-Hill, New York (1983) pp 731-747.

50. Steinberg D, Herndon Jr JH, Uhlendorf BW, Mize CE, Avigan J, Milne GWA. Refsum's disease: Nature of the enzyme defect. Science **156**, 1740-1742 (1967).

51. Pettersen JE, Jellum E, Eldjarn L. The occurence of adipic and suberic acid in urine from ketotic patients. Clin Chim Acta **38**, 17-24 (1972).

52. Wada F, Usami M, Goto M, Hayashi T. Studies on the physiological significance of fatty acid ω-oxidation. J Biochem **70**, 1065-1067 (1971).

53. Björkhem I. On the mechanism of regulation of ω-oxidation of fatty acids. J Biol Chem **251**, 5259-5266 (1976).

54. Parvin R, Pande SV. Enhancement of mitochondrial carnitine and carnitine acylcarnitine translocase-mediated transport of fatty acids into liver mitochondria under ketogenic conditions. J Biol Chem **254**, 5423-5429 (1979).

55. McGarry JD, Foster DW. Regulation of hepatic fatty acid oxidation and ketone body production. Ann Rev Biochem **49**, 395-420 (1980).

56. Mortensen PB. Dicarboxylic acids and the lipid metabolism. Danish Med Bull **31**, 121-145 (1984).

57. Schmidt JE. Medical discoveries. CC Thomas, Springfield (1959) p 193.

58. Wolkow M, Baumann E. Ueber das Wesen der Alkaptonurie. Hoppe Seyler's Z Physiol Chem **15**, 228-285 (1891).

59. Følling A. Über Ausscheidung von Phenylbrenztraubensäure in den Harn als Stoffwechselanomalie in Verbindung mit Imbezillität. Hoppe-Seyler's Z Physiol Chem **227**, 169-176 (1934).

60. Harris H. Garrod's Inborn Errors of Metabolism. Oxford University Press, London (1963).

61. Eldjarn L, Jellum E, Stokke O. Inborn errors of metabolism - a challenge to the clinical chemist. Clin Chem **21**, 63-66 (1975).

62. Tanaka K, Budd MA, Efron ML, Isselbacher KJ. Isovaleric acidemia: a new genetic defect of leucine metabolism. Proc Nat Acad Sci USA **56**, 236-242 (1966).

63. Oberholzer VG, Levin B, Burgess EA, Young WF. Methylmalonic aciduria. An inborn error of metabolism leading to chronic metabolic acidosis. Arch Dis Childh **42**, 492-504 (1967).

64. Hommes FA, Kuipers JRG, Elema JD, Jansen JF, Jonxis JHP. Propionicacidemia, a new inborn error of metabolism. Pediat Res **2**, 519-524 (1968).

65. Eldjarn L, Stokke O, Jellum E. Pyroglutamic aciduria. A new inborn error of metabolism possibly in the "γ-Glutamyl cycle" proposed for amino acid transport. In: Organic Acidurias. J Stern, C Toothill, Eds., Churchill Livingstone, Edinburgh (1972) pp 113-120.

66. Stokke O, Jellum E, Eldjarn L. A new metabolic error in the leucine degradation pathway: β-hydroxyisovaleric aciduria and β-methylcrotonylglycinuria. In: Organic Acidurias. J Stern, C Toothill, Eds., Churchill Livingstone, Edinburgh (1972) pp 27-34.

67. Gompertz D, Draffan GH. Tiglyl glycine excretion in a child with β-methylcrotonyl glycinuria. In: Organic Acidurias. J Stern, C Toothill, Eds., Churchill Livingstone, Edinburgh (1972) pp 35-37.

68. Schreier K, Porath U. Kongenitale metabolische Acidosen in der Postnatalperiode. Dtsch med Wschr **103**, 943-947 (1978).

69. Burton BK, Nadler HL. Clinical diagnosis of the inborn errors of metabolism in the neonatal period. Pediatrics **61**, 398-405 (1978).

70. Chalmers RA, Purkiss P, Watts RWE, Lawson AM. Screening for organic acidurias and amino acidopathies in newborns and children. J Inher Metab Dis **3**, 27-43 (1980).

71. Sweetman L. Prenatal diagnosis of the organic acidurias. J Inher Metab Dis **7**, Suppl. 1, 18-22 (1984).

72. Morrow III G, Schwarz RH, Hallock JA, Barness LA. Prenatal detection of methylmalonic acidemia. J Pediatr **77**, 120-123 (1970).

73. Zinn AB, Hine DG, Mahoney MJ, Tanaka K. The stable isotope dilution method for measurement of methylmalonic acid: a highly accurate approach to the prenatal diagnosis of methylmalonic acidemia. Pediatr Res **16**, 740-745 (1982).

74. Fensom AH, Benson PF, Chalmers RA, Tracey BM, Watson D, King GS, Pettit BR, Rodeck CH. Experience with prenatal diagnosis of propionic acidaemia and methylmalonic aciduria. J Inher Metab Dis **7**, Suppl. 2, 127-128 (1984).

75. Dancis J. Maple syrup urine disease. In: Antenatal Diagnosis. A Dorfman, Ed., University of Chicago Press, Chicago (1972) pp 123-125.

76. Wendel U, Rüdiger HW, Passarge E, Mikkelsen M. Maple syrup urine disease: rapid prenatal diagnosis by enzyme assay. Humangenetik **19**, 127-128 (1973).

77. Gompertz D, Goodey PA, Thom H, Russell G, Johnston AW, Mellor DH, MacLean MW, Ferguson-Smith ME, Ferguson-Smith MA. Prenatal diagnosis and family studies in a case of propionic acidaemia. Clin Genet **8**, 244-250 (1975).

78. Naylor G, Sweetman L, Nyhan WL, Hornbeck C, Griffiths J, Mörch L, Brandänge S. Isotope dilution analysis of methylcitric acid in amniotic fluid for the prenatal diagnosis of propionic and methylmalonic acidemia. Clin Chim Acta **107**, 175-183 (1980).

79. Duran M, Schutgens RBH, Ketel A, Heymans H, Berntssen MWJ, Ketting D, Wadman SK. 3-Hydroxy-3-methylglutaryl coenzyme A lyase deficiency: postnatal management following prenatal diagnosis by analysis of maternal urine. J Pediatr **95**, 1004-1007 (1979).

80. Chalmers RA, Tracey BM, Mistry J, Stacey TE, McFadyen IR, Madigan MJ. The prenatal diagnosis of 3-hydroxy-3-methylglutaric aciduria by GC-MS and enzymology on cultured amniocytes and on chorionic villi. Pediatr Res **19**, 1075 (1985).

81. Goodman SI, Gallegos DA, Pullin CJ, Halpern B, Truscott RJW, Wise G, Wilcken B, Ryan ED, Whelen DT. Antenatal diagnosis of glutaric aciduria. Am J Hum Genet **32**, 695-699 (1980).

82. Gangné R, Lescault A, Grenier A, Laberge C, Mélançon SB, Dallaire L. Prenatal diagnosis of tyrosinaemia: measurement of succinylacetone in amniotic fluid. Prenat Diag **2**, 185-188 (1982).

83. Kvittingen EA, Steinmann B, Gitzelmann R, Leonard JV, Andria G, Børresen AL, Mossman J, Micara G, Lindblad B. Prenatal diagnosis of hereditary tyrosinemia by determination of fumarylacetoacetase in cultured amniotic fluid cells. Pediatr Res **19**, 334-337 (1985).

84. Jakobs C, Kvittingen EA, Berger R, Haagen A, Kleijer W, Niermeijer M. Prenatal diagnosis of tyrosineamia type I by use of stable isotope dilution mass spectrometry. Eur J Pediatr **144**, 209-210 (1985).

85. Hoffmann F. Fundamenta Medicinae. LS King, Ed.,Science History Publications, New York (1971) pp 83-87.

86. Jellum E, Stokke O, Eldjarn L. Screening for inborn errors of metabolism using gas-liquid chromatography, mass spectrometry and computer technique. In: Organic Acidurias. J Stern, C Toothill, Eds., Churchill Livingstone, Edinburgh (1972) pp 167-174.

87. Herman JA, Suttle JF. Precipitation and crystallization. In: Treatise on Analytical Chemistry I:3. IM Kolthoff, PJ Elving, Eds., Interscience Publishers (1961) pp 1367-1409.

88. Zlatkis A, Lichtenstein HA, Tishbee A, Bertsch W, Shunbo F, Liebich HM. Concentration and analysis of volatile urinary metabolites. J Chrom Sci **11**, 299-302 (1973).

89. Matsumoto KE, Partridge DH, Robinson AB, Pauling L, Flath RA, Mon TR, Teranishi R. The identification of volatile compounds in human urine. J Chromatogr **85**, 31-34 (1973).

90. Kettrup A. Gas chromatographic headspace analysis: its theory and applications. Trends Anal Chem **1**, 142-145 (1982).

91. Buchanan DN, Thoene JG. Volatile carboxylic acid profiling in physiological fluids: direct injection into a gas chromatograph/mass spectrometer. Clin Chim Acta **145**, 183-191 (1985).

92. Irving H, Williams RJP. Liquid-liquid extraction. In: Treatise on Analytical Chemistry **I:3**. IM Kolthoff, PJ Elving, Eds., Interscience Publishers (1961) pp 1309-1365.

93. Jellum E. Profiling of human body fluids in healthy and diseased states using gas chromatography and mass spectrometry, with special reference to organic acids. J Chromatogr **143**, 427-462 (1977).

94. Chalmers RA, Lawson AM. Organic Acids in Man. Analytical chemistry, biochemistry and diagnosis of the organic acidurias. Chapman & Hall, London (1982).

95. Swim HE, Utter MF. Isotopic experimentation with intermediates of the tricarboxylic acid cycle. Methods in Enzymology **IV**, 584-609 (1957).

96. Bachmann C, Bühlmann R, Colombo JP. Organic acids in urine: sample preparation for GC/MS. J Inher Metab Dis **7**, Suppl. 2, 126 (1984).

97. Rosenthal I, Weiss AR, Usdin VR. Chromatography: general principles. In: Treatise on Analytical Chemistry **I:3**. IM Kolthoff, PJ Elving, Eds., Interscience Publishers (1961) pp 1411-1467.

98. Billiet H, Laurent C, de Galan L. A reappreciation of alumina in high-performance liquid chromatography. Trends Anal Chem **4**, 100-103 (1985).

99. Thompson JA, Markey SP. Quantitative metabolic profiling of urinary organic acids by gas chromatography-mass spectrometry: comparison of isolation methods. Anal Chem **47**, 1313-1321 (1975).

100. Boujet C, Drouet C, Decoux G, Favier A. Etude de l'extraction des acides organiques urinaires. Etape prealable à leur séparation en chromatographie en phase gazeuse. J Chromatogr **228**, 67-74 (1982).

101. Eriksson A, Lindstedt S, Ransnäs L, von Wendt L. Deficiency of glycerol kinase (EC 2.7.1.30). Clin Chem **29**, 718-722 (1983).

102. Greter J, Holme E, Lindstedt S, Koivikko M. Biotin-responsive 3-methylcrotonylglycinuria with biotinidase deficiency. J Inher Metab Dis **8**, Suppl. 2, 103-104 (1985).

103. Willstätter R. Untersuchungen über Enzyme I. J Springer Verlag, Berlin (1928) pp 66-67.

104. Kuhn R, Winterstein A, Lederer E. Zur Kenntnis der Xanthophylle. Hoppe-Seyler's Z Physiol Chem **197**, 141-160 (1931).

105. Heftmann E. Chromatography. 2nd ed., Reinhold Publishing Corp., New York (1967).

106. Karger BL, Snyder LR, Horvath C. An Introduction to Separation Science. John Wiley & Sons, New York (1973).

107. Consden R, Gordon AH, Martin AJP. Qualitative analysis of proteins: a partition chromatographic method using paper. Biochem J **38**, 224-232 (1944).

108. Stahl E. Dünnschicht-Chromatographie. 2nd ed., Springer-Verlag, Berlin (1967).

109. Hostettmann K. Neuere Chromatographie. Verfahren für die Isolierung und Trennung von Naturstoffen. Naturwissenschaften **70**, 186-189 (1983).

110. Sherma J, Fried B. Thin-layer and paper chromatography. Anal Chem **56**, 48R-63R (1984).

111. James AT, Martin AJP. Gas-liquid partition chromatography: the separation and micro-estimation of volatile fatty acids from formic acid to dodecanoic acid. Biochem J **50**, 679-690 (1952).

112. Desty DH. Capillary columns: trials, tribulations, and triumphs. Adv Chromatogr **1**, 199-228 (1965).

113. Kaiser R. Chromatographie in der Gasphase **1**. Bibliographisches Institut, Mannheim (1973).

114. Kaiser RE. Chromatographie in der Gasphase **2**. Bibliographisches Institut, Mannheim (1975).

115. Karasek FW, Onuska FI, Yang FJ, Clement RE. Gas chromatography. Anal Chem **56**, 174R-199R (1984).

116. Schwarzenbach R. High-performance liquid chromatography of carboxylic acids. J Chromatogr **251**, 339-358 (1982).

117. Majors RE, Barth HG, Lochmüller CH. Column liquid chromatography. Anal Chem **56**, 300R-349R (1984).

118. Horning EC, Horning MG. Metabolic profiles: gas-phase methods for analysis of metabolites. Clin Chem **17**, 802-809 (1971).

119. Street HV. The use of gas-liquid chromatography in clinical chemistry. Adv Clin Chem **12**, 217-307 (1969).

120. German AL, Pfaffenberger CD, Thenot J-P, Horning MG, Horning EC. High resolution gas chromatography with thermostable glass open tubular capillary columns. Anal Chem **45**, 930-935 (1973).

121. Ettre LS. Open-tubular columns: evolution, present status, and future. Anal Chem **57**, 1419A-1438A (1985).

122. Jennings W. Gas Chromatography with Glass Capillary Columns. Academic Press, New York (1978).

123. Grob K, Grob G. Static Coating: the solvent;the source of vacuum. J High Res Chrom Chrom Commun **8**, 856-857 (1985).

124. Madani C, Chambaz EM, Rigaud M, Durand J, Chebroux P. New method for the preparation of highly stable polysiloxane-coated glass open-tubular capillary columns and application to the analysis of hormonal steroids. J Chromatogr **126**, 161-169 (1976).

125. Madani C, Chambaz EM. Glass open-tubular capillary columns with chemically bonded methyl-phenyl siloxane stationary phases of tailor made polarity. Chromatographia **11**, 725-730 (1978).

126. Grob K, Grob G. Capillary columns with immobilized stationary phases. II. Practical advantages and details of procedure. J Chromatogr **213**, 211-221 (1981).

127. Wright BW, Peaden PA, Lee ML, Stark TJ. Free radical cross-linking in the preparation of non-extractable stationary phases for capillary gas chromatography. J Chromatogr **248**, 17-34 (1982).

128. Schomburg G, Husmann H, Ruthe S, Herraiz M. Crosslinking of alkylpolysiloxane films on various types of glass surfaces including fused silica using γ-radiation of ^{60}cobolt-source. Comparison to crosslinking by thermal peroxid treatment. Chromatographia **15**, 599-610 (1982).

129. Blomberg LG, Markides KE. The role of organosilicon chemistry in the preparation of capillary columns for gas chromatography. J High Res Chrom Chrom Commun **8**, 632-650 (1985).

130. Jentzsch D, Hövermann W. The application of golay columns of larger internal diameter. J Chromatogr **11**, 440-451 (1963).

131. Teranishi R, Mon TR. Large-bore capillary and low-pressure-drop packed columns. Anal Chem **36**, 1490-1492 (1964).

132. Grob K, Grob G, Blum W, Walther W. Preparation of inert glass capillary columns for gas chromatography. A revised, comprehensive description. J Chromatogr **244**, 197-208 (1982).

133. Hippocrates. Prognostic **XII** and Aphorisms **IV**: LXIX-LXXXIII, **VII**:XXXI-XXXV. WHS Jones, Ed., W Heinemann, London (1923 and 1953).

134. Nachmanson E. Ur de Medicinska Upptäckternas Historia. Bonniers Förlag, Stockholm (1943) p 111.

135. Woodruff SD, Yeung ES. Refractive index and absorption detector for liquid chromatography based on Fabry-Perot Interferometry. Anal Chem **54**, 1174-1178 (1982).

136. Bennett MJ, Bradey CE. Simpler liquid-chromatographic screening for organic acid disorders. Clin Chem **30**, 542-546 (1984).

137. Liebes LF. Continuous-flow scanning of selected high-performance liquid chromatography peak conponents by microprocessor control. Application to analysis of extracts from human lymphocytes. J Chromatogr **219**, 255-262 (1981).

138. Mattiuz EL, Webb JW, Gates SC. High resolution separation of urinary organic acids by high performance liquid chromatography. J Liq Chromatogr **5**, 2343-2357 (1982).

139. DiCesare JL, Ettre LS. New ways to increase the specificity of detection in liquid chromatography. J Chromatogr **251**, 1-16 (1982).

140. Gunasingham H, Fleet B. Use of electrochemical detection in normal-phase high-performance liquid chromatography. J Chromatogr **261**, 43-53 (1983).

141. Haddad PR, Alexander PW, Trojanowicz M. High-performance liquid chromatography of organic acids with potentiometric detection using a metallic copper electrode. J Chromatogr **315**, 261-270 (1984).

142. Keulemans AIM. Gas Chromatography. CG Verver, Ed., Reinhold Publishing Corp., New York (1957).

143. Harley J, Nel W, Pretorius V. Flame ionization detector for gas chromatography. Nature **181**, 177-178 (1958).

144. McWilliam IG. The origin of the flame ionization detector. Chromatographia **17**, 241-243 (1983).

145. Thomason MM, Bertsch W, Apps P, Pretorius V. A simple device for optimization of flame ionization detectors and determination of absolute sensitivity. J High Res Chrom Chrom Commun **5**, 690-692 (1982).

146. Albertyn DE, Bannon CD, Craske JD, Hai NT, O'Rourke KL, Szonyi C. Analysis of fatty acid methyl esters with high accuracy and reliability. I. Optimization of flame-ionization detectors with respect to linearity. J Chromatogr **247**, 47-61 (1982).

147. Bennett CE, Nogare SD, Safranski LW, Lewis CD. Trace analyses by gas chromatography. Anal Chem **30**, 898-902 (1958).

148. Wells G, Simon R. Thermal conductivity detector: theory and numerical model. J Chromatogr **256,** 1-15 (1983).

149. Poole CF. The electron-capture detector in capillary column gas chromatography. J High Res Chrom Chrom Commun **5,** 454-471 (1982).

150. Freedman AN. Photoionization detector response. J Chromatogr **236,** 11-15 (1982).

151. Seelemann R. GC/FTIR coupling - a modern tool in analytical chemistry. Trends Anal Chem **1,** 333-339 (1982).

152. Griffiths PR, de Haseth JA, Azarraga LV. Capillary GC/FT-IR. Anal Chem **55,** 1381A-1387A (1983).

153. McDonald RS. Infrared Spectrometry. Anal Chem **56,** 349R-372R (1984).

154. Beynon JH. 80 years of mass spectrometry. Trends Anal Chem **1,** 292-295 (1982).

155. Svec HJ. Mass spectroscopy - ways and means. A historical prospectus. Int J Mass Spectrom Ion Proc **66,** 3-29 (1985).

156. Roboz J. Introduction to Mass Spectrometry. Instrumentation and techniques. Interscience Publishers, New York (1968).

157. McFadden W. Techniques of Combined Gas Chromatography/Mass Spectrometry: Applications in Organic Analysis. John Wiley & Sons, New York (1973).

158. Schlunegger UP. Practical aspects and trends in analytical organic--mass spectrometry. Topics Curr Chem **95,** 49-88 (1981).

159. Porter CJ, Beynon JH, Ast T. The modern mass spectrometer-a complete chemical laboratory. Org Mass Spectrom **16,** 101-114 (1981).

160. Burlingame AL, Whitney JO, Russell DH. Mass spectrometry. Anal Chem **56,** 417R-467R (1984).

161. Todd JFJ. A survey of the current state of quadrupole mass spectrometry. Int J Mass Spectrom Ion Proc **60,** 3-10 (1984).

162. McLafferty FW. Trends in analytical instrumentation. Science **226,** 251-253 (1984).

163. Budzikiewicz H, Djerassi C, Williams DH. Mass Spectrometry of Organic Compounds. Holden-Day, San Francisco (1967).

164. Milne GWA. Mass Spectrometry: Techniques and Applications. Wiley-Interscience, New York (1971).

165. Hamming MC, Foster NG. Interpretation of Mass Spectra of Organic Compounds. Academic Press, New York (1972).

166. Roboz J. Mass spectrometry in clinical chemistry. Adv Clin Chem **17**, 109-191 (1975).

167. McLafferty FW. Interpretation of Mass Spectra. 3rd ed., University Science Books, Mill Valley (1980).

168. Caprioli RM, Liehr JG, Seifert Jr WE. Applications to clinical medicine. In: Biochemical Applications of Mass Spectrometry. 1st suppl. vol., GR Waller, OC Dermer, Eds., John Wiley & Sons, New York (1980).

169. Howe I, Williams DH, Bowen RD. Mass spectrometry. Principles and applications. 2nd ed., McGraw-Hill, New York (1981).

170. Morris HR. Biomolecular structure determination by mass spectrometry. Nature **286**, 447-452 (1980).

171. Halpern B. Biomedical application of gas chromatography-mass spectrometry. CRC Crit Rev Anal Chem **11**, 49-78 (1981).

172. Cooks RG, Busch KL, Glish GL. Mass spectrometry: analytical capabilities and potentials. Science **222**, 273-291 (1983).

173. Bŏcek P, Janák J. Flame ionisation detection. (Flame ionisation phenomena). Chromatogr Rev **15**, 111-150 (1971).

174. Kauffmann H, Knapp O, Straub H. Ein FID neuer Bauform. Auswirkungen auf die Nachweiseigenschaften. Chromatographia **5**, 558-563 (1972).

175. Karasek FW. GC/MS/Computers. Anal Chem **44**, 32A-42A (1972).

176. Goodman SI, Markey SP. Diagnosis of Organic Acidemias by Gas chromatography-Mass Spectrometry. AR Liss, New York (1981).

177. Budzikiewicz H. Massenspektrometrie negativer Ionen. Angew Chem **93**, 635-649 (1981).

178. Dougherty RC. Negative chemical ionization mass spectrometry. Anal Chem **53**, 625A-636A (1981).

179. Richter WJ, Schwarz H. Chemische Ionisation - ein stark an Bedeutung gewinnendes massenspectrometrisches Analysenverfahren. Angew Chem **90**, 449-469 (1978).

180. Cotter RJ. Mass Spectrometry of nonvolatile compounds. Desorption from extended probes. Anal Chem **52**, 1589A-1606A (1980).

181. Rinehart Jr KL. Fast atom bombardment mass spectrometry. Science **218**, 254-260 (1982).

182. Martin SA, Costello CE, Biemann K. Optimization of experimental procedures for fast atom bombardment mass spectrometry. Anal Chem **54**, 2362-2368 (1982).

183. Röllgen FW. Field desorption mass spectrometry. Trends Anal Chem **1**, 304-307 (1982).

184. Marcuson R, Burbeck SL, Emond RL, Latter GI, Aberth W. Normalization and reproducibility of mass profiles in the detection of individual differences from urine. Clin Chem **28**, 1346-1348 (1982).

185. Horning EC, Horning MG, Carroll DI, Dzidic I, Stillwell RN. New picogram detection system based on a mass spectrometer with an external ionization source at atmospheric pressure. Anal Chem **45**, 936-943 (1973).

186. Vestal ML, Fergusson GJ. Thermospray liquid chromatograph/mass spectrometer interface with direct electrical heating of the capillary. Anal Chem **57**, 2373-2378 (1985).

187. Schmelzeisen-Redeker G, McDowall MA, Giessmann U, Levsen K, Röllgen FW. Studies with a laboratory-constructed thermospray liquid chromatograph-mass spectrometer interface. J Chromatogr **323**, 127-133 (1985).

188. Henneberg D, Henrichs U, Schomburg G. Special techniques in the combination of gas chromatography and mass spectrometry. J Chromatogr **112**, 343-352 (1975).

189. Henneberg D, Henrichs U, Husmann H, Schomburg G. High-performance gas chromatograph-mass spectrometer interfacing: investigation and optimization of flow and temperature. J Chromatogr **167**, 139-147 (1978).

190. Andresen BD, Ng KJ, Wu J, Bianchine JR. Helium preheater for the open-split interface. Biomed Mass Spectrom **8**, 237-239 (1981).

191. Wetzel E, Kuster Th. Construction of a gas chromatography-mass spectrometry interface using a fused-silica transfer line. J Chromatogr **268**, 177-185 (1983).

192. Holland JF, Enke CG, Allison J, Stults JT, Pinkston JD, Newcome B, Watson JT. Mass spectrometry on the chromatographic time scale: realistic expectations. Anal Chem **55**, 997A-1012A (1983).

193. Kram TC, Cooper DA, Allen AC. Behind the indentification of China White. Anal Chem **53**, 1379A-1381A, 1384A, 1386A (1981).

194. Cheng MT, Kruppa GH, McLafferty FW, Cooper DA. Structural information from tandem mass spectrometry for China White and related fentanyl derivatives. Anal Chem **54**, 2204-2207 (1982).

195. Jellum E. Gas chromatography-mass spectrometry in diagnosis of human metabolic disease. Trends Anal Chem **1**, 12-16 (1981).

196. Vestal ML. High-performance liquid chromatography-mass spectrometry. Science **226,** 275-281 (1984).

197. Arpino PJ. Ten years of liquid chromatography-mass spectrometry. J Chromatogr **323,** 3-11 (1985).

198. Alborn H, Stenhagen G. Direct coupling of packed fused-silica liquid chromatographic columns to a magnetic sector mass spectrometer and application to polar thermolabile compounds. J Chromatogr **323,** 47-66 (1985).

199. Jellum E, Stokke O, Eldjarn L. Combined use of gas chromatography, mass spectrometry, and computer in diagnosis and studies of metabolic disorders. Clin Chem **18,** 800-809 (1972).

200. Brown GK, Stokke O, Jellum E. Chromatographic profile of high boiling point organic acids in human urine. J Chromatogr **145,** 177-184 (1978).

201. Lewis S, Kenyon CN, Meili J, Burlingame AL. High resolution gas chromatographic/real-time high resolution mass spectrometric identification of organic acids in human urine. Anal Chem **51,** 1275-1285 (1979).

202. Spiteller M, Spiteller G. Trennung und Charakterisierung saurer Harnbestandteile. J Chromatogr **164,** 253-317 (1979).

203. Spiteller M, Spiteller G. Über das Auftreten α-alkyl-substituierter Äpfelsäuren und β-hydroxy-β-alkyl-substituierter Dicarbon- und Tricarbonsäurederivate als normale Stoffwechselprodukte. J Chromatogr **164,** 319-329 (1979).

204. Grupe A, Spiteller G. New polar acid metabolites in human urine. J Chromatogr **226,** 301-314 (1981).

205. Jellum E. Multi-component analyses of human body fluids and tissues in health and disease using capillary gas chromatography-mass spectrometry and high-resolution two-dimensional electrophoresis. J Chromatogr **239,** 29-37 (1982).

206. Tanaka K, Hine DG. Compilation of gas chromatographic retention indices of 163 metabolically important organic acids, and their use in detection of patients with organic acidurias. J Chromatogr **239,** 301-322 (1982).

207. Tuchman M, Bowers LD, Fregien KD, Crippin PJ, Krivit W. Capillary gas chromatographic separation of urinary organic acids. Retention indices of 101 urinary acids on a 5% phenylmethyl silicone capillary column. J Chromatogr Sci **22,** 198-202 (1984).

208. Bauer S, Spiteller G. Strukturaufklärung und Synthese bisher unbekannter Furancarbonsäuren aus Humanurin. Liebigs Ann Chem **1985,** 813-821 (1985).

209. Heindl A, Rau O, Spiteller G. Identification of aromatic dihydroxy acids in biological fluids. Biomed Mass Spectrom **12**, 59-66 (1985).

210. Spiteller G. Kombination chromatographischer Trennmethoden mit der Massenspektrometrie - ein modernes Verfahren zur Stoffwechseluntersuchung. Angew Chem **97**, 461-476 (1985).

211. Chapman JR. Computers in Mass Spectrometry. Academic Press, New York (1978).

212. Jellum E, Helland P, Eldjarn L, Markwardt U, Marhöfer J. The development of a computer-assisted search for anomalous compounds (CASAC). J Chromatogr **112**, 573-580 (1975).

213. Dromey RG, Stefik MJ, Rindfleisch TC, Duffield AM. Extraction of mass spectra free of background and neighboring component contributions from gas chromatography/mass spectrometry data. Anal Chem **48**, 1368-1375 (1976).

214. Blaisdell BE, Gates SC, Martin FE, Sweeley CC. Comparison of two methods of detection, determination, and library search of the substances present in gas chromatography-mass spectrometry data. Anal Chim Acta **117**, 35-43 (1980).

215. Martinsen DP. Survey of computer aided methods for mass spectral interpretation. Applied Spectroscopy **35**, 255-266 (1981).

216. Smith DH, Gray NAB, Nourse JG, Crandell CW. The dendral project: recent advances in computer-assisted structure elucidation. Anal Chim Acta **133**, 471-497 (1981).

217. Vrbanac JJ, Sweeley CC, Pinkston JD. Automated metabolic profiling analysis of urinary steroids by a gas chromatography mass spectrometry data system. Biomed Mass Spectrom **10**, 155-161 (1983).

218. Domokos L, Henneberg D, Weimann B. Computer-aided identification of compounds by comparison of mass spectra. Anal Chim Acta **165**, 61-74 (1984).

219. Wold S, Christie OHJ. Extraction of mass spectral information by a combination of autocorrelation and principal components models. Anal Chim Acta **165**, 51-59 (1984).

220. McLafferty FW, Stauffer DB. Retrieval and interpretative computer programs for mass spectrometry. J Chem Inf Comput Sci **25**, 245-252 (1985).

221. Rosenthal D. Theoretical limitations of gas chromatographic/mass spectrometric identification of multicomponent mixtures. Anal Chem **54**, 63-66 (1982).

222. Ende M, Pfeifer P, Spiteller G. Über das Einschleppen von Verunreinigungen bei Verwendung von Extrelut®-Fertigsäulen. J Chromatogr **183**, 1-7 (1980).

223. Ende M, Spiteller G. Contaminants in mass spectrometry. Mass Spectrom Rev **1**, 29-62 (1982).

224. Franich RA. Ester artefacts formed during isolation of ω-hydroxy fatty acids by DEAE-Sephadex ion-exchange chromatography. J Chromatogr **168**, 538-540 (1979).

225. Bauer S, Neupert M, Spiteller G. Characterization of artefacts produced by treatment of organic acids with diazomethane. J Chromatogr **309**, 243-259 (1984).

226. Fales HM, Comstock W, Jones TH. Test for dehydrogenation in gas chromatography-mass spectrometry systems. Anal Chem **52**, 980-982 (1980).

227. van Slyke DD, Palmer WW. Studies of acidosis. XVI. The titration of organic acids in urine. J Biol Chem **41**, 567-585 (1920).

228. Winkler-Oswatitsch R, Eigen M. Die Kunst zu Titrieren. Vom klassischen Endpunktverfahren zur modernen differentiellen und dynamischen Analyse. Angew Chem **91**, 20-51 (1979).

229. Peele Jr JD, Gadsden RH, Crews R. Semi-automated vs. visual reading of urinalysis dipsticks. Clin Chem **23**, 2242-2246 (1977).

230. Sweeley CC, Elliott WH, Fries I, Ryhage R. Mass spectrometric determination of unresolved components in gas chromatographic effluents. Anal Chem **38**, 1549-1553 (1966).

231. Samuelsson B, Hamberg M, Sweeley CC. Quantitative gas chromatography of prostaglandin E_1 at the nanogram level: use of deuterated carrier and multiple-ion analyzer. Anal Biochem **38**, 301-304 (1970).

232. Millard BJ. Quantitative Mass Spectrometry. Heyden, London (1978).

233. Lehmann WD, Schulten H-R. Quantitative Massenspektrometrie in Biochemie und Medizin. Angew Chem **90**, 233-250 (1978).

234. Hites RA, Biemann K. Computer evaluation of continuously scanned mass spectra of gas chromatographic effluents. Anal Chem **42**, 855-860 (1970).

235. Reimendal R, Sjövall J. Analysis of steroids by off-line computerized gas chromatography-mass spectrometry. Anal Chem **44**, 21-29 (1972).

236. Büttner J, Borth R, Broughton PMG, Bowyer RC. Quality control in clinical chemistry. J Clin Chem Clin Biochem **18**, 535-541 (1980).

237. ACS Subcommittee on Environmental Analytical Chemistry. Guidelines for data acquisition and data quality evaluation in environmental chemistry. Anal Chem **52,** 2242-2249 (1980).

238. Lawson AM, Gaskell SJ, Hjelm M. Methodological aspects on quantitative mass spectrometry used for accuracy control in clinical chemistry. J Clin Chem Clin Biochem **23,** 433-441 (1985).

239. Milne GWA, Budde WL, Heller SR, Martinsen DP, Oldham RG. Quality control and evaluation of mass spectra. Org Mass Spectrom **17,** 547-552 (1982).

240. Jonckheere JA, De Leenheer AP, Steyaert HL. Statistical evaluation of calibration curve nonlinearity in isotope dilution gas chromatography/mass spectrometry. Anal Chem **55,** 153-155 (1983).

241. Khots MS, Kogan DL. Precision characteristics of low-resolution mass spectra of organic compounds. Zhurnal Analiticheskoi Khimii **38,** 1851-1855 (1983).

242. Low IA, Liu RH, Barker SA, Fish F, Settine RL, Piotrowski EG, Damert WC, Liu JY. Selected ion monitoring mass spectrometry: parameters affecting quantitative determination. Biomed Mass Spectrom **12,** 633-637 (1985).

243. Smith DH, Achenbach M, Yeager WJ, Anderson PJ, Fitch WL, Rindfleisch TC. Quantitative comparison of combined gas chromatographic/mass spectrometric profiles of complex mixtures. Anal Chem **49,** 1623-1632 (1977).

244. Ryan JF, Gebhart JE, Rando LC, Perry DL, Tomer KE, Pellizzari ED, Bursey JT. The master analytical scheme: an assessment of factors influencing precision and accuracy of gas chromatography/mass spectrometry data. In: Advances in the Identification & Analysis of Organic Pollutants in Water **1.** LH Keith, Ed., Ann Arbor Science Publishers, Ann Arbor (1981) pp 49-66.

245. Flachs H, Larsen E, Schroll G. Intercomparison of 11 mass spectrometers applied for quantitative organic analysis in Denmark. Biomed Mass Spectrom **11,** 545-548 (1984).

246. Lawson AM, Chalmers RA, Watts RWE. Urinary organic acids in man. I. Normal patterns. Clin Chem **22,** 1283-1287 (1976).

247. Chalmers RA, Healy MJR, Lawson AM, Watts RWE. Urinary organic acids in man. II. Effects of individual variation and diet on the urinary excretion of acidic metabolites. Clin Chem **22,** 1288-1291 (1976).

248. Chalmers RA, Healy MJR, Lawson AM, Hart JT, Watts RWE. Urinary organic acids in man. III. Quantitative ranges and patterns of excretion in a normal population. Clin Chem **22,** 1292-1298 (1976).

249. Robertson WG, Hodgkinson A, Marshall DH. Seasonal variations in the composition of urine from normal subjects: a longitudinal study. Clin Chim Acta **80,** 347-353 (1977).

250. Davis BA, Boulton AA. Longitudinal urinary excretion of some "trace" acids in a human male. J Chromatogr **222,** 161-169 (1981).

251. Krause K-H, Kochen W, Berlit P, Bonjour J-P. Excretion of organic acids associated with biotin deficiency in chronic anticonvulsant therapy. Internat J Vit Nutr Res **54,** 217-222 (1984).

252. Mamer OA, Montgomery JA, Deckelbaum RJ, Granot E. Identification of urinary 3-ethoxy-4-hydroxybenzoic and 3-ethoxy-4-hydroxymandelic acid after dietary intake of ethyl vanillin. Biomed Mass Spectrom **12,** 163-169 (1985).

253. Pscheidt GR, Berlet HH, Spaide J, Himwich HE. Variations of urinary creatinine and its correlation to excretion of indole metabolities in mental patients. Clin Chim Acta **13,** 228-234 (1966).

254. Applegarth DA, Hardwick DF, Ross PM. Creatinine excretion in children and the usefulness of creatinine equivalents in amino acid chromatography. Clin Chim Acta **22,** 131-134 (1968).

255. Tocci PM, Phillips J, Sager R. The effect of diet upon the excretion of parahydroxyphenylacetic acid and creatinine in man. Clin Chim Acta **40,** 449-453 (1972).

256. Witten TA, Levine SP, King JO, Markey SP. Gas-Chromatographic mass-spectrometric determination of urinary acid profiles of normal young adults on a controlled diet. Clin Chem **19,** 586-589 (1973).

257. Narayanan S, Appleton HD. Creatinine: a review. Clin Chem **26,** 1119-1126 (1980).

258. Vestergaard P, Leverett R. Constancy of urinary creatinine excretion. J Lab Clin Med **51,** 211-218 (1958).

259. Cramér K, Cramér H, Selander S. A comparative analysis between variation in 24-hour urinary creatinine output and 24-hour urinary volume. Clin Chim Acta **15,** 331-335 (1967).

260. Banda PW, Tuttle MS, Sherry AE, Blois MS. Total creatinine content of the first morning urine is independent of dietary change. Clin Chem **26,** 535-536 (1980).

261. Mizuno T, Abe N, Teshima H, Yamauchi E, Itagaki Y, Matsumoto I, Kuhara T, Shinka T. Application of gas chromatography mass spectrometry computer system for clinical diagnosis. Biomed Mass Spectrom **8,** 593-597 (1981).

262. Hirschfeld T. The hy-phen-ated methods. Anal Chem **52,** 297A, 299A, 300A, 302A, 304A, 308A, 312A (1980).

263. Wilkins CL, Giss GN, Brissey GM, Steiner S. Direct-linked gas chromatography-fourier transform infrared-mass spectrometer system. Anal Chem **53,** 113-117 (1981).

GÖTEBORGS UNIVERSITET
GÖTEBORGS UNIVERSITETSBIBLIOTEK