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Separation, quantification and identification of non-volatile organic acids in body fluids by gas chromatography

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Abstract: The present paper concentrates on the analysis of non-volatile organic acids in physiological fluids. The solvent extraction and DEAE-Sephadex extraction of organic acids, respectively, seem to be the most widely used methods for isolation of this group of compounds from the biological matrix. Gas chromatography–mass spectrometry (GC/MS) is the preferred method for the separation, quantification and identification. In three tables organic acids are divided into the classes according to a functional group, a survey of alkylation methods is given and retention indices of common urinary organic acids on various GC columns are summarized.

Keywords: *Non-volatile organic acids; capillary gas chromatography; mass spectrometry; metabolite disorders; organic acidurias; biological sample preparation.*

Introduction

Analyses of organic acids as metabolic products of endogenous as well as exogenous substances excreted and contained in various body fluids (urine, blood plasma, serum, amniotic fluid, cerebro-spinal fluid, tissue homogenates, etc.), are involved in many diagnostic processes in clinical biochemistry, pharmacology and toxicology. Diagnosis of inherited metabolic disorders belongs to the most characteristic and most complicated areas where various analyses of organic acids are concentrated: (a) analysis of excreted organic acids and their concentrations as data characterizing human metabolism and pathological state variations; (b) analysis of metabolites of medicaments given during therapy of pathological states; (c) analysis of excreted substances in acute pathological states corresponding to metabolic disorders but caused by intoxication with exogenous substances.

Part of the diagnosis of inherited metabolic disorders is the analysis of more groups of substances: organic and amino acids, purines and pyrimidines, oligosaccharides, neurotransmitters and other similar substances. Analysis of organic acids may be further divided according to the properties of the substance to be analyzed and, therefore, according to the methodologies used in analysis of volatile organic acids, non-volatile organic acids and their conjugates and fatty acids.

This paper presents a short review on the classification of organic acids and then concentrates on the analysis of non-volatile organic acids, which is the largest group concerned in the diagnosis of inherited metabolic disorders. The material presented here is also applicable to other fields because of similarity in structures and properties of the organic acids concerned.

Those concerned in fields not discussed here may refer to papers dealing with analysis of volatile organic acids [1–3], fatty acids, [4, 5] and conjugates of organic acids [6, 7].

A preferred method for separation of organic acids is gas chromatography (GC), although this method can only be used for substances which are volatile at temperatures below approximately 300°C, either directly or after conversion to suitable derivatives. This method can also be combined in a simple manner with mass spectrometry (MS), mostly used for identification of individual components of a mixture of organic acids. Other methods of analysis, e.g. HPLC, TLC, HPTLC, isotachophoresis, are only mentioned here with references to pertinent papers dealing with these methods and their applications in greater detail.

Finally, it would be suitable to define in the introductory part the term organic acid as it is used in this paper: organic acids are all carboxylic acids which may contain further functional groups such as oxo, hydroxyl, methoxyl and others, plus heterocycles. Amino acids are not included although other acids containing nitrogen are, e.g. pyroglutamic acid, conjugates with amino acids. Common features of these acids are high solubility in water, acidity and negative reaction with ninhydrin.

Classification of organic acids in physiological fluids

More than 500 organic acids have been found in urine and other biological fluids; approximately 250 acids were identified, mainly using GC/MS method. The most extensive papers are those by M. Spiteller and G. Spiteller [8–10] and the paper by Grupe and G. Spitteler [11], which reports on identification and GC/MS data for approximately 230 organic acids.

Organic acids found in body fluids can be divided into various groups from various viewpoints. Since this paper is essentially concerned with extraction, separation and determination methods, classification according to functional groups being connected with the behaviour of these substances in extraction and separation has been adopted (Table 1). Other divisions of organic acids can be found, e.g. in the survey by Liebich [12].

Extraction of organic acids from physiological fluids

Extraction of organic acids from aqueous medium must be carried out prior to their separation using gas chromatography as conversion to volatile derivatives (methyl, trimethylsilyl TMS) requires non-aqueous medium.

Suitable sample treatment — deproteinization, acidification, saturation with a suitable salt, usually NaCl, etc. are carried out prior to the extraction, depending on the type of fluid under examination.

Organic acids are usually extracted from urine. Early morning urine (EMU) is used and less often, 24-h urine. It is necessary to freeze urine immediately after collection if possible without preservatives in order to prevent formation of artifacts. When

Table 1
Classes of organic acids in serum and urine

Class	Functional group (other than carboxylic)	Number of C atoms in molecule	Example
1. Carboxyl only containing			
a. short chain	—	1–5	volatile fatty acids
b. medium and long chain	—	6 and more	fatty acids
c. dicarboxylic	—	2–18	saturated and unsaturated dicarboxylic acids
d. tricarboxylic	—	6	aconitic acid
2. Hydroxy acids			
a. monohydroxy	-OH	2–16	
b. polyhydroxy	2 and more OH	3–6	
c. hydroxy dicarboxylic	-OH	4–14	
d. hydroxy tricarboxylic	-OH	6–8	citric acid
e. acid lactones	lactone	5–6	hydroxy acid lactones
3. Keto acids	=O	2–11	
a. monocarboxylic keto acids	=O	2–11	
b. dicarboxylic keto acids	=O	5–6	
		heteroatomes/ heterocycles in molecule	
4. Nitrogen, sulphur or heterocycle			
a. sulphur containing	$-SCH_3$, =O	S	4-methylthio-2-keto-butyric acid
b. glycine containing	-NH-	N	glycine conjugates
c. glutamate containing	-NH-	N	acylglutamic acids
d. glutamine containing	-NH-, $-CONH_2$	2 × N	acylglutamines
e. amino group containing	$-NR_2$, $-NH_2$	N	*N*-acetylaminoacids anthranilic acid
f. pyrrolidine containing	-NH-	pyrrolidine	pyroglutamic acid
g. pyridine containing	-N=	pyridine	nicotinic acid
h. imidazole containing	-NH-, -N=	imidazole	imidazollactic acid
i. indole containing	-NH-	indole	3-indolelactic acid
j. chinoline containing	-N=, -OH	chinoline	kynurenic acid
k. furancarboxylic	-O—	furane	furan-2,5-dicarboxylic acid
l. purine containing	4x -NH-, 2x = O	purine	uric acid
m. pyrimidine containing	2x -NH—, 2x = O	pyrimidine	orotic acid

immediate freezing is not possible, some workers use ascorbic acid, merthiolate [*o*-(carboxyphenyl)thioethyl mercury] and other substances such as preservatives. Urine frozen to −20°C or less can be kept till sample analysis. Urine should be transported in dry ice (solid carbon dioxide).

Some other protein-free fluids like saliva and sweat, are rarely used in addition to urine for determination of organic acids.

Deproteinization procedure

From protein-containing physiological fluids, organic acids are mostly determined in serum, plasma, cerebrospinal fluid (SF), amniotic fluid (AF) or in tissue homogenates.

Plasma should be preferred over serum and whole blood [13], although whole blood has also been used for determinations [14]. Variations in concentrations or composition of some acids may occur during the time required for the blood to clot. Plasma should be taken in heparin tubes rather than using heparin solution [15], separated by centrifugation or frozen to −20°C.

Deproteinization of these physiological fluids should be carried out as soon as possible as the enzymes contained therein can affect the levels of some acids. For deproteinization prior to extraction of organic acids as a group, acid deproteinization agents (trichloroacetic acid, sulphosalicylic acid, perchloric acid) cannot be used as they may interfere analysis and derivatization.

Deproteinization with ethanol [16, 17] and ultrafiltration [18], respectively, are usually used.

After deproteinization, subsequent extraction is performed in the same manner as in the case of protein-free fluids.

Two processes are generally used for extraction of organic acids from biological fluids: solvent extraction and extraction with DEAE-Sephadex. The extraction processes are

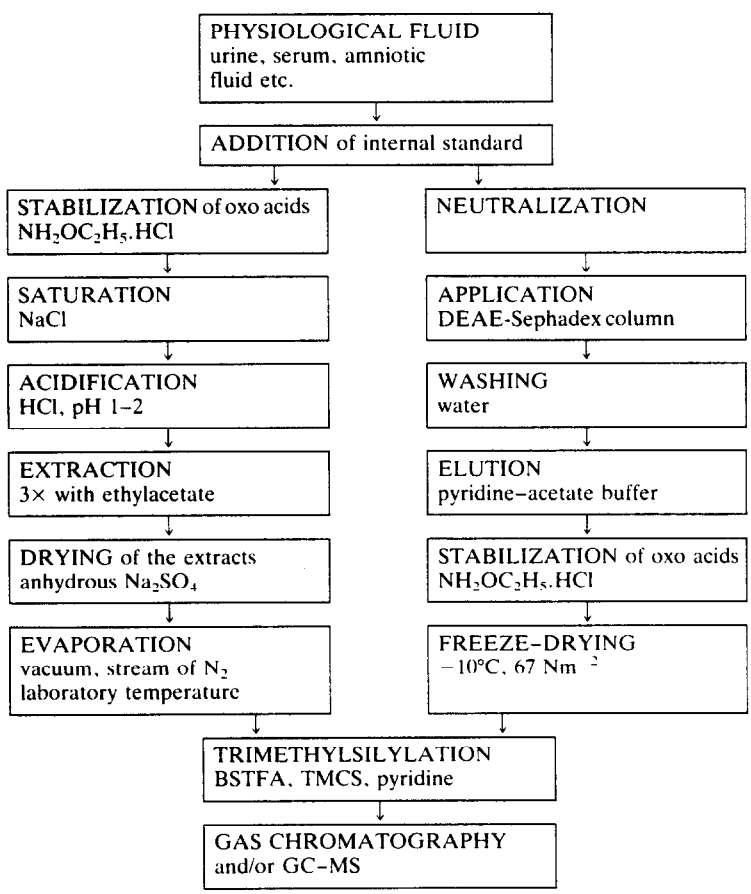


Figure 1
Extraction of the organic acids using solvent extraction or DEAE-Sephadex extraction.

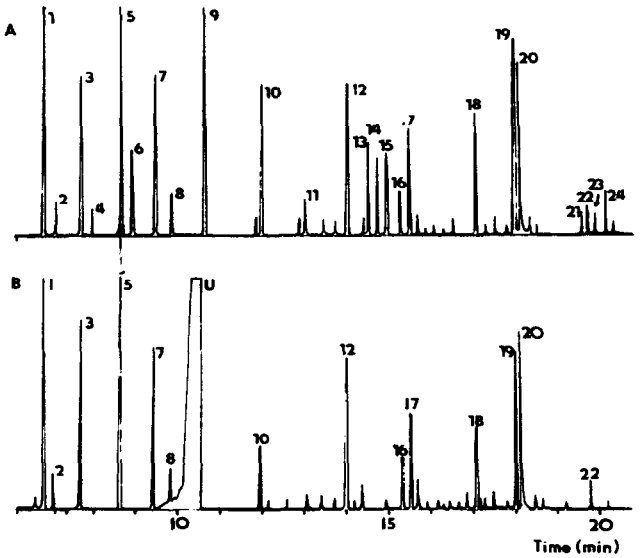


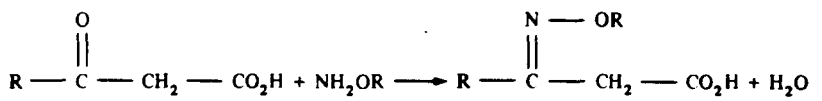
Figure 2
Chromatogram of urine organic acids obtained by GC analysis on BP-5 (bonded SE-54 phase) FSOT column using the following temperature program: 2 min at 80°C, 10°C × min $^{-1}$ to 270°C, 5 min at 270°C. Organic acids were extracted using DEAE-Sephadex (A) and ethylacetate (B), ethoximated and trimethylsilylated.

Peak identifications are: 1-lactic acid di TMS; 2-glycolic acid di TMS; 3-pyruvic acid-ethoxime mono TMS; 4-oxalic acid di TMS; 5-3-hydroxybutyric acid di TMS; 6-sulfuric acid di TMS; 7-malonic acid di TMS (internal standard), 8-3-hydroxyisovaleric acid di TMS; 9-phosphoric acid tri TMS; 10-4-deoxytetronic acid (threono) tri TMS; 11-2-deoxytetronic tri TMS; 12-adipic acid di TMS; 13-erythronic acid tetra TMS; 14-threonic acid tetra TMS; 15-2-hydroxyglutaric tri TMS; 16-3-hydroxy-3-methylglutaric tri TMS; 17-2-oxoglutaric acid ethoxime di TMS; 18-aconitic acid tri TMS; 19-citric acid tetra TMS; 20-hippuric acid di TMS; 21-galactonic acid hexa TMS; 22-palmitic acid mono TMS; 23-glucuronic acid penta TMS; 24-glucaric acid hexa TMS; U-urea di TMS.

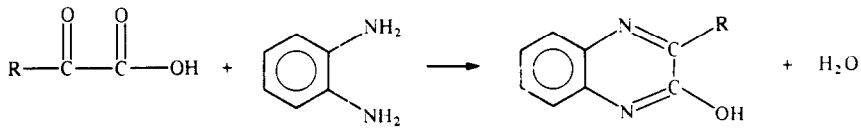
schematically shown in Fig. 1, comparison of chromatograms obtained with GC analysis of organic acids isolated from urine using both processes is given in Fig. 2.

Stabilization of oxo acids

In extraction and GC analysis, oxo acids such as TMS derivatives may cause some problems — they undergo oxidative decarboxylation during extraction, e.g. phenyl pyruvate → phenyl acetate, or show two peaks in the chromatogram as the result of keto-enol tautomerization. Therefore, these acids are stabilized prior to further treatment by conversion to oximes [19], methoximes [20] ethoximes [21, 22] or other *O*-substituted oximes [23]. The reaction proceeds as follows:



Aliphatic 2-oxo acids can be converted by reaction with *o*-phenylene diamine to quinoxalinols [24] as follows:



Solvent extraction

This method is now mostly used because of its simplicity and rate although some acids are extracted with low yield (especially groups 1d, 2b, c, d and 4f, i, l in the Table 1). Ethyl acetate extraction [25–27], stepwise extraction with ethyl acetate and ether [28] or extraction with a mixture of these two solvents [29] are mostly used. Other solvents also have been tested in order to improve yields of extraction, especially of hydrophylic acids: methyl acetate [22], isopropyl ether [30], isopropyl chloride [31], tri-(*n*-butyl)phosphate [32].

Solvent extraction is mostly performed from an acidified sample (pH value 1–2) saturated with salt (NaCl, Na_2SO_4) at room temperature. Continuous extraction from hot acidified urine has been used as well to improve yields [33, 34]. However, the increased temperature can lead to the formation of artifacts.

The solvents used for extraction usually dissolve a certain amount of water also. In this way, many interfering substances may pass into the extracted sample: phosphates, sulphates, urea, etc. Since water interferes with derivatization, extracts are often dried by addition of anhydrous Na_2SO_4. However, some authors claim that losses of organic acids occur [35, 36] and recommend that residues be dried after evaporation of solvents by addition of a few drops of benzene and reevaporated.

Extraction with DEAE-Sephadex

This method gives substantially higher extraction yields, especially in cases of acids where solvent extraction is ineffective (e.g. glyceric, erythronic, threonic 1,3- and 4-hydroxyacetic and other acids [29]). It is suitable especially for extraction of organic acids as a whole group in deeper study of metabolism and metabolic disorders.

This process was originally put forward by Jaakonmaki *et al.* [37]. It has been refined and intensively used by Horning and Horning [38, 39], and later on by many other authors [40–43]. Thompson and Markey modified the extraction process so to remove, by means of precipitation, barium hydroxide sulphates and phosphates which are found in high concentrations, especially in urine, interfere with determinations and can reduce the life of chromatographic columns [44]. However, Lawson *et al.* claim that barium hydroxide precipitation may cause co-precipitation of some acids leading to losses in extraction [22]; therefore, this modification is still being discussed [45, 46].

DEAE-Sephadex extraction proceeds as follows: Neutral deproteinized sample is introduced onto the column of DEAE-Sephadex (diethylaminoethyl ion exchange group on dextran gel) where the acids are retained whereas neutral and basic substances are eluted. Acids are eluted, mostly with pyridine–acetate aqueous buffer, stabilization of oxo acids is performed as in solvent extraction and the sample is lyophilized. When precipitation of SO_4^{2-} and PO_4^{3-} salt is performed, it is done so prior to introduction onto the column by addition of BaOH solution at a pH value higher than 11. Sample lyophilization requires adhering to certain conditions in order to prevent losses of more volatile acids. Chalmers and Watts [47] mention the requirement of pressure 67 N m^{-2} (0.5 torr) and temperature −10°C. In order to prevent losses of acids, elution with HCl

and subsequent neutralization with $NaHCO_3$ to produce the less volatile sodium salts has also been proposed [48].

Comparison of isolation techniques

Selection of the most suitable methods requires appreciation of their advantages and disadvantages for certain applications. Extraction with DEAE-Sephadex is more time consuming since sample lyophilization usually proceeds overnight requiring at least 10 h. Also maintenance of the lyophilization conditions (temperature, pressure) is not usually possible when using available lyophilization devices so that it is necessary to modify the device or to use a homemade device [49]. Solvent extraction is relatively simple and fast, sample treatment does not usually require more than 1–1.5 h. On the other hand, quantitative results cannot be obtained for many acids.

With respect to these disadvantages of both commonly used extraction methods, further procedures have been worked out. Some of them are successfully applied now in many laboratories.

Alternative isolation procedures

Extraction methods using non-specific sorbents have been developed as alternatives to solvent extraction. Bachman *et al.* [50] describe, for instance, a method for sample preparation for GC/MS analysis based on adsorption of acids on a silica gel column (the authors used the column Extrelut Merck) and subsequent elution with a mixture of chloroform/tertiary amyl alcohol with re-extraction of acids using up to 1 mol l^{-1} of aqueous ammonia which was then evaporated in a stream of N_2. The yields of the extraction process are given by the authors ranging between 84 and 116%. Anderson and Fitch [51] proposed application of cellulose (extraction test tubes JETUBES, Manhattan Instr., USA) for adsorption of acids from urine with subsequent elution using the mixture ether/ethyl acetate 1 : 1. Alcock [52] attained good result with a two-stage process using silica gel and Celite in the first stage, aluminium oxide in the second. Acids are eluted using ether in the first stage and methanol in the second stage at simultaneous methylation with BF_3 which participates as a catalyst.

Methods using other types of ion exchangers have been developed as an alternative to extraction with DEAE-Sephadex. In early dealing with the analysis of organic acids, Dowex 1 (tetramethyl ammonium ion on styrene–divinyl benzene matrix) was used for extraction [53]. Later on, a micromethod for determination [54] was elaborated as a modification of the mentioned process which is now used for extraction of citrate cycle acids (e.g. 55). Zaura and Metcoff [56] used Dowex 2 (trimethyl hydroxyethyl ammonium ion on matrix STY-DVB) for extraction in formate cycle with elution of acids using formic acid followed by evaporation of the eluate in vacuum before derivatization. The reported extraction yield ranges between 37 and 127%.

Nakamura [57] used Dowex 3 (polyamine ion on STY-DVB matrix) for extraction of dicarboxylic acids and reported an extraction yield of about 87%. On the other hand, some authors state that possible decomposition of some acids should be anticipated on strong anion exchange resins [58, 59].

Recently, an interesting method for extraction with Dowex 1 with subsequent *in situ* methylation of acids using methyl iodide was described [60]. This method is a modification of extractive methylation when acids are extracted as an ion pair with tetramethyl ammonium hydroxide and, after extraction, methylated with methyl iodide using TMAH as a reaction catalyst [61]. The method using Dowex 1 is based on the same

principle except that TMAH ions are bonded to the ion-exchanger matrix. In contrast to the original extractive methylation [62] when the extraction yield is very dependent on the pH value, as a narrow pH range in the region of pK of the acid is necessary for formation of an ion pair, the extractive methylation on an ion-exchanging resin can be used for extraction and methylation of organic acids as a group. The yield of this procedure is in the range of 75–110%.

Derivatives of carboxylic and hydroxyacids

Organic acids of interest within the scope of the study of metabolic disorders are usually relatively highly polar substances as they often contain, in addition to the carboxylic group, functional groups which increase their polarity (see Table 1). They have low volatility and this renders them unsuitable for gas chromatography.

Organic acids, with the exception of volatile short-chain fatty acids with C_1–C_5, should therefore, be converted to more volatile derivatives. The usual derivatives are methyl esters and trimethylsilyl (TMS) esters and ethers.

An advantage of methyl ester derivatives is their stability in the presence of water or air humidity whereas TMS derivatives are sensitive to hydrolysis. Methyl ester derivatives have lower molecular weights (which is advantageous for mass spectrometry), higher volatility, more interpretable mass spectra and, finally, they contaminate FID electrodes to a lesser degree than TMS derivatives, as silicon is formed during the combustion of the latter compounds.

On the other hand, TMS derivatives have many advantages: easy and quantitative preparation using safe-to-handle and ready-to-use reagents, less side reactions during preparation, etc. Due to these advantages, TMS derivatives are now preferred by many workers.

Methyl derivatives of organic acids

Methyl derivatives are often prepared by methylation with diazomethane prepared most suitably from Diazald (*N*-methyl-*N*-nitroso-*p*-toluene-sulphonamide) [63] or other precursors using reaction with KOH. The diazomethane is either directly introduced into the sample or absorbed in cooled ether and used for methylation in the form of a solution. A disadvantage is that the diazomethane solution prepared in this way cannot be kept for a long time, not even in a freezing box.

Methylation with diazomethane proceeds as follows:

$$R—COOH + CH_2{=}N{=}N \longrightarrow R—COOCH_3 + N_2$$

and is sometimes called permethylation as not only esterification of carboxylic groups, but also methylation of phenolic OH group proceeds. There have been observed many further reactions: non-quantitative methylation of alcoholic OH groups, partial methylation of amino groups [64], diazomethane addition to double bond e.g. of fumaric acid and aconitic acid under formation of pyrazolines [65, 66], etc. Some authors recommend reaction at −70°C in order to prevent side reactions, especially addition across double bonds [67, 68].

Large disadvantages of methylation with diazomethane are its toxicity, explosive instability and potential carcinogenicity of its precursors (for this reason, it is safest to use Diazald). Therefore, other methods for preparation of methyl esters and, in general,

Table 2
Alkylation methods for organic acid derivatization

Reagent	Solvent	Catalyst	Derivative	Ref.
N,N-dimethylformamide dialkylacetals	Pyridine, benzene, chloroform etc.	—	Methyl, ethyl, propyl, butyl	113
Dimethyl sulphate	Water	K_2CO_3	Methyl	114
Methanol	—	*N,N*-dicyclohexylcarbodiimide	Methyl	115
Alcohol	—	Mineral acid. Org. acid anhydride	Various alkyl	
Alcohol	—	BF_3	Various alkyl	
Alkyl halide	Aprotic, e.g. acetone, CH_2Cl_2	Basic agents	Various alkyl	61
Alkyl halide	pentane	$AgNo_3$	Various alkyl	116, 117
Trimethylanilinium hydroxide	Methanol, water	Pyrolysis	Methyl	118

alkyl esters have been used as given in Table 2. A characteristic chromatogram of urinary organic acids after conversion to methyl esters is given in Fig. 3.

TMS derivatives of organic acids

TMS esters and ethers are mostly prepared using bis-trimethylsilyl trifluoroacetamide (BSTFA) or *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA) by means of reaction of acids with the reagent in a suitable solvent e.g. pyridine, chloroform or without solvent. Reaction may be preferably catalyzed by addition of trimethyl chlorosilane (TMCS) or pyridine and performed at room temperature or at 40–50°C. When preparing the sample for derivatization during the reaction, sensitivity of silylating agents as well as TMS derivatives to water traces and air humidity should be borne in mind. The reaction samples should therefore be well dried and protected from moisture using suitable reaction vessels, e.g. with PTFE closure enabling addition of reagents by means of a dry microsyringe.

Using this method, carboxylic groups of acids, alcoholic and phenolic –OH groups and NH groups in *N*-acyl glycines may be derivatized. The latter compounds are not, however, quantitatively silylated and usually give two peaks in chromatograms. Silylation of sodium salts of organic acids is sometimes difficult and use of BSTFA in a mixture with TMCS and/or hydroxylamine enables quantitative yields to be obtained [69].

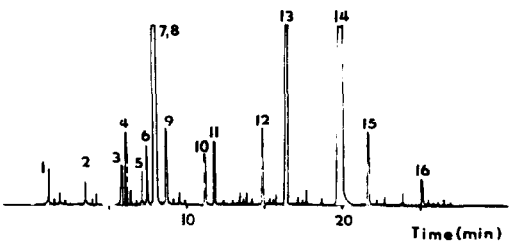


Figure 3
Chromatogram of urine organic acids obtained by GC analysis on BP-10 (7% cyanopropyl, 7% phenyl methyl siloxane bonded phase) FSOT column following temperature program: 2 min at 30°C, 8°C per min to 230°C, 5 min at 230°C. Organic acids were extracted using ethylacetate and derivatized by diazomethane.

Peak identifications are: 1-3-hydroxybutyric acid mono M; 2-oxalic acid di M; 3-methylmalonic acid di M; 4-cresol mono M; 5-ethylmalonic acid di M; 6-succinic acid di M; 7-cresol not derivatized, 8-benzoic acid mono M; 9-citraconic acid di M; 10-adipic acid di M; 11-3-methyladipic acid di M; 12-4-hydroxyphenylacetic acid di M; 13-citric acid 3 M; 14-hippuric acid tri M; 15-3-indoleacetic acid di M; 16-5-hydroxyindoleacetic acid tri M.

Other derivatives of organic acids

Besides alkyl derivatives and TMS derivatives, some other derivatives of organic acids have been proposed. For instance, methylation of carboxylic groups of acids with diazomethane (reaction time 1 min) was used with subsequent silylation of hydroxy groups with mixtures of HMDS and TMCS [70, 71]. Attempts have been made to avoid non-quantitative methylation of hydroxy groups in alcohols when using diazomethane but these mixed derivatives have now been overcome by application of TMS-only derivatives.

Some authors have studied the application of acylating agents for derivatization of –OH and –NH groups with subsequent methylation [72–74]. However, application of

trifluoroacetylation, tetrafluorobutyrylation or acetylation did not lead to quantitative results.

Lately, application of alkyl dimethylsilyl derivatives, especially tertiary butyl dimethylsilyl derivatives was proposed because of their high stability in the presence of water [75]. The authors state that the stability of these derivatives is approximately 10^4-fold higher than that of TMS derivatives and these derivatives may be separated using TCL. An advantage in mass spectrometry is that *t*-BDMS derivatives give intensive $[M - (t\text{-butyl})]^+$ ion which can be advantageously used for determinations of organic acids using the single ion monitoring (SIM) method [76, 77].

Separation, quantification and identification of organic acids

Separation of organic acids

Firstly, it is necessary to use a suitable separation method for quantification and identification of individual organic acids in an intricate mixture obtained by extraction of biological fluids. Most often, this separation is performed by means of gas chromatography although sometimes other separation methods such as TLC, HPLC and column liquid chromatography are used. Details relating to these latter methods can be found in reviews on applications of TLC [78, 79] and in papers on applications of HPLC [80–82] in the field of analysis of organic acids.

Advantages of gas chromatography (GC) include the possibility to use this method for the separation of various groups of substances, with relatively high separation efficiency (especially when using capillary or high resolution gas chromatography, HRGC), simplicity in comparison with other chromatographic techniques, reproducibility, possibility of simple connection to a mass spectrometer as a high-quality identification unit or other selective detectors, etc. With respect to these advantages, gas chromatography is used for determination of the so-called organic acid profiles as well as for determination of limited groups of organic acids in detailed studies of metabolic processes.

Separation may be, in principle, performed using two procedures:

1. After extraction of organic acids from the body fluid and conversion to volatile derivatives, the separation conditions are selected so that the maximum data of all contained acids may be obtained in a single determination.
2. After extraction and derivatization, only a limited group of acids is determined.

The number of acid substances in urine is estimated to be approximately 500, more than 250 have been partially or fully identified. The efficiency of packed columns is not able to cope with the full separation of so many substances. Therefore, all forms of modern high resolution gas chromatography, HRGC, are utilized for determination of urinary organic acid profiles. On the other hand, inborn errors of metabolism are characterized by relatively substantial increase in concentration of characteristic metabolites so that these substances can be separated and approximately quantified with less efficient systems [83]. For that reason, some institutions still continue to use packed columns in gas chromatography analysis. For separation of organic acids in urine and biological fluids, readily available commercial capillary columns are often used, usually fused silica open tubular columns (FSOT) having length of 25–100 m and internal diameter of 0.25–0.5 mm. From the viewpoint of time necessary for analysis, short columns with smaller internal diameters may be used to obtain higher separating efficiency (commercial columns 12 m × 0.1 mm i.d.). However, the choice of a suitable

column depends on many factors which should be considered in each particular case. Details are beyond the scope of this paper and publications dealing with this theme in the field of HRGC and HRGC/MS [84–87] are recommended.

Silicones OV-101, SE-30, SE-54 are often used as non-polar stationary phase on inner capillary surface and OV-1701 as a medium-polar phase. Application of the so-called crosslinked and bonded phases is advantageous, especially in HRGC/MS [89, 89] when free radicals are formed in the phase either chemically (organic peroxides) or by gamma radiation after coating the inner surface with a suitable silicone so that reactions between polymer molecules (crosslinking) and between polymer molecules and hydroxyl groups on the capillary surface (bonding) proceed. This results, after rinsing of the excess stationary phase with a suitable solvent, in columns with very small liquid phase bleeding suitable for working with detectors sensitive to contamination such as MS, for working with programmable temperature when only small increase in bleeding at higher temperatures occurs. When working with acid samples extracted from biological fluids, they also have the advantage that the columns contaminated with larger volume of samples can be rinsed with a suitable solvent.

These stationary phases can be used also for separation of optical isomers of organic acids after conversion to diastereoisomers by means of reaction with suitable chiral reagents [90–92]. Separation of optical isomers on chiral stationary phases as is often used with amino acids is not commonly used in separation of organic acids.

A requirement of stationary phases used for analysis of organic acids is stable up to 300–320°C for determination of some less volatile compounds. Separation of the overall spectrum is achieved using analysis with temperature programming. The choice of the temperature rise rate is often a compromise between the time necessary for analysis and the separation quality.

The sample is usually injected by the split stream method as the concentration of the substances of interest in the sample is usually sufficient. On-column injection has the advantage of smaller sample fractionation and leads to better separation of low-boiling components at the expense of high-boiling ones. At the same time, this method leads to considerable plugging of columns with other components of the biological material.

An advantageous alternative is the so-called fast split injection [93] when injection is made using an automatic device with injection time of approximately 100 ms. According to the author, proportioning and fractionation accuracy is nearly the same as in the case of injection onto the packed columns so that it is possible to use a single internal standard even in case of analysis of broad-boiling range mixtures.

Quantification of organic acids is carried out using an internal standard or determination of relative response factor (RRF). In the first case, a known volume of a suitable substance is added to urine sample (usually according to creatinine content in urine); this substance may be an organic acid non-occurring in biological samples (e.g. 3-chlorophenylacetic acid, tropic acid, diphenylacetic acid, malonic acid). Calculated peak areas of the analyzed substances are related to the internal standard area, concentrations of substances of interest are calculated by analysis of a calibration mixture treated by the same procedure as the analyzed sample. An advantage of this method is the fact that the internal standard is added at the beginning of the sample preparation and therefore, affected by the same treatment conditions.

In the case of quantification using RRF, the RRF value is experimentally determined for each substance in relation to the standard substance added before analysis (e.g. hydrocarbons C_{24} or C_{26}). A disadvantage of this method is that extraction variability is

not accounted for in the result obtained and that the relation between RRF and the sample concentration is not always linear over the whole range of interest [43]. An advantage is that RRF values for relatively large amount of substances are published in the literature and these values can be usually used directly [94, 43].

A special modification of the internal standard method is determination using the single ion monitoring (SIM) method for organic acids in a sample with added isotope-labelled internal standard. Deuterium is usually used for labelling, the labelled substance has the same chemical properties and is extracted in the same way as the non-labelled substance. Also the chromatographic properties are the same so that the substance to be determined and its labelled internal standard elute in a single chromatographic peak; when using the SIM method in GC/MS, quantification of each component can be performed separately. The described method enables the most exact determination of important organic acids and is mostly used for determination of their concentrations in amniotic fluid in pre-natal diagnostics of organic acidurias [95, 96].

A detailed report on this method and a survey of its applications in biochemistry can be found in the literature [97, 98].

Identification of organic acids is performed either by means of chromatographic methods as a preliminary identification method e.g. for screening of organic acidurias or, more often, by GC/MS.

In case of identification using retention data obtained by gas chromatography analysis, the so-called methylene units (MU) are generally used which are calculated for the given substance as follows: analysis of a standard mixture of *n*-alkanes is performed simultaneously with the sample under conditions used for analysis of the investigated sample. From the retention times of the hydrocarbon standards, their carbon number and the retention time of the compound of interest an MU value may be assigned. The MU value of a substance which elutes at half time between C_{11} and C_{12} *n*-alkane is assigned, in this way, as 11.5. Identification of materials can be substantially more precise when the MU values are established on two columns with phases having different polarities. This method was used by Tanaka [99] who published the MU values for a large number of organic acids separated in columns with packing of OV-1 as a non-polar phase and OV-17 as a medium-polar phase. Recently, MU values for organic acids analyzed in various capillary columns were published. The published MU values for the individual acids are given in Table 3.

Values of retention times relative to those of the internal standard or material used for RRF calculation (mostly C_{24} n-alkane) have also been used for identification of organic acids (e.g. 100). Identification of organic acids is faster, simpler and substantially more reliable when using mass spectrometry.

Many spectra of organic acids are available in various mass spectra libraries often in the form of databases. Computer programs which are also a part of the data system are used for comparison of sample spectra with the spectra in the library. Efficient computers enable very quick comparison of spectra so that the comparison of the spectrum of the substance searched for with those stored in the library does not require usually more than 10 to 30 s if 40 to 70,000 spectra are stored in the library. Despite these advantages provided by the commercial data systems, many institutions dealing with analysis of organic acids use their own identification (and quantification) programs and often use for identification the MU values of the substance in addition to mass spectra. Also a library of mass spectra is often compiled in the institutions by means of analysis of standards of organic acids, and of samples of biological fluids from patients. These

Table 3
Retention indices of common urinary organic acids

Compound	Retention index methylene units				
	DB-1*	Ultra-2*	DB-1701*	OV-1‡	OV-17‡
Lactic di TMS	10.61	10.66	11.16	10.58	10.95
2-Hydroxyisobutyric di TMS	—	10.71	—	10.66	10.74
Glycolic di TMS	10.73	10.81	11.45	10.70	11.27
Glyoxylic-EO-mono TMS	—	—	—	10.55§	—
Glyoxylic-oxime di TMS	11.20	11.35	11.91	11.21	11.96
n-Caproic mono TMS	10.69	—	11.24	10.63	11.24
Oxalic di TMS	11.19	11.39	12.38	11.21	12.29
2-Hydroxybutyric di TMS	11.29	11.36	11.80	11.30	11.61
p-Cresol mono TMS	11.42	11.54	12.16	11.38	12.38
Pyruvic-EO-mono TMS	—	—	—	11.04§	—
Pyruvic oxime di TMS	11.48	11.58	12.04	11.47	12.11
3-hydroxypropionic di TMS	11.42	11.51	12.07	11.40	11.94
2-oxoisovaleric-EO-mono TMS, pk 1	—	—	—	11.40§	—
Dipropylacetic mono TMS	11.56	12	12.02	11.47	11.87
2-Oxobutyric-EO mono TMS	—	—	—	11.52	—
3-Hydroxybutyric di TMS	11.64	11.67	12.15	11.60	11.94
3-Hydroxysobutyric di TMS	11.70	11.70	12.16	11.63	11.96
Acetoacetic-EO-mono TMS, pk 1	—	—	—	11.64§	—
2-Hydroxyisovaleric di TMS	11.71	11.74	12.13	11.70	11.88
2-Oxo-3-methylvaleric-EO mono TMS, pk 1	—	—	—	11.86§	—
2-Methylacetoacetic-EO mono TMS, pk1	—	—	—	11.88§	—
Acetoacetic-EO mono TMS, pk 2	—	—	—	11.88§	—
2-Oxobutyric oxime di TMS	11.88	—	12.40	11.88	12.46
3-hydroxy-2-methylbutyric di TMS	12.00	12.05	12.48	12.09	12.25
3-Hydroxyisovaleric di TMS	12.16	12.16	12.58	12.14	12.35
2-Oxoisovaleric oxime di TMS	12.09	—	12.58	12.13	12.69
Methylmalonic di TMS	12.09	12.25	13.02	12.12	12.86
Urea di TMS	12.30	12.49	14.65	12.25	13.50
2-Oxocaproic-EO mono TMS	—	—	—	12.25§	—
2-Oxo-3-methylvaleric-EO mono TMS, pk 2	—	—	—	12.28§	—
Benzoic mono TMS	12.33	12.49	13.45	12.28	13.73
2-Ethylhydracrylic di TMS	12.31	—	12.81	12.33	12.63
2-Hydroxyisocaproic di TMS	12.38	—	12.84	12.41	12.61
3-Hydroxyvaleric di TMS	12.38	—	12.86	12.39	12.70
2-Hydroxy-3-methylvaleric di TMS	12.47	—	12.88	12.44§	—
2-Ethylacetoacetic-EO mono TMS, pk 1	—	—	—	12.50§	—
2-Oxovaleric oxime di TMS	12.52	—	13.05	12.55	13.05
Acetylglycine mono TMS	12.53	—	15.06	12.53	14.89
Octanoic mono TMS	12.63	—	13.19	12.56	13.14
2-Oxoisocaproic-EO mono TMS, pk 1 and 2	—	—	—	12.56§	—
2-ethylacetoacetic-EO mono TMS, pk 2	—	—	—	12.62§	—
2-Hydroxycaproic di TMS	12.82	—	13.30	12.84	13.10
2-Oxo-3-methylvaleric L-oxime di TMS	12.70	—	13.13	12.73	13.24
Phosphoric tri TMS	12.73	12.89	13.90	12.76	13.46
Ethylmalonic di TMS	12.77	—	13.66	12.78	13.48
Maleic di TMS	12.96	—	14.16	12.93	14.21

Table 3
(Continued)

Compound	Retention index methylene units				
	DB-1*	Ultra-2*	DB-1701*	OV-1‡	OV-17‡
Phenylacetic mono TMS	12.82	13.05	14.06	12.77	14.37
2-Oxoisocaproic oxime di TMS	12.86	—	13.31	12.89	13.34
2-Oxo-3-methylvaleric D-oxime di TMS	12.88	—	13.41	12.88	13.33
Glycerol tri TMS	12.92	—	12.61	12.92	12.63
Succinic di TMS	13.08	13.22	14.10	13.08	14.02
Thymol mono TMS	13.12	—	13.73	13.10	13.88
Methylensuccinic di TMS	13.23	—	14.18	13.21	14.60
2-Oxocaproic oxime di TMS	13.31	—	13.80	13.32	13.85
4-Oxovaleric-EO mono TMS	—	—	—	13.38§	—
Propionylglycine mono TMS	13.30	13.73	15.83	13.34	15.37
Glyceric tri TMS	13.41	13.45	13.89	13.43	13.60
Fumaric di TMS	13.44	13.54	14.38	13.49	14.03
4-Deoxytetronic/erythro/tri TMS	—	13.60	—	13.82§	—
4-Deoxytetronic/threo/no/tri TMS	—	13.69	—	13.88§	—
Acetylglycine di TMS	13.59	—	15.17	13.57	14.86
Glutaric di TMS	13.91	14.10	15.02	13.96	14.87
Propionylglycine di TMS	—	—	—	14.17	15.37
3-Methylglutaric di TMS	14.16	—	15.17	14.19	15.01
n-Decanoic mono TMS	14.51	14.50	15.09	14.43	15.06
3-Deoxytetronic tri TMS	—	14.26	—	14.43§	—
2-Deoxytetronic tri TMS	—	14.47	—	14.64§	—
Isovalerylglycine mono TMS	15.05	15.02	17.03	14.64	16.56
Mandelic di TMS	14.73	14.91	15.77	14.71§	—
Pyroglutamic di TMS	15.10	15.36	17.05	15.07	16.80
Adipic di TMS	14.97	15.14	16.12	14.99	15.97
Malic tri TMS	14.98	15.05	15.79	15.01	15.39
Trans-3-dihydromucoic di TMS	15.03	—	16.31	15.06	16.22
Isovalerylglycine di TMS	14.58	15.29	16.26	15.10	16.02
Salicylic di TMS	15.07	—	16.17	15.05	16.32
2-Oxo-4-methiolbutyric-EO mono TMS	—	—	—	15.00§	—
2-Oxo-4-methiolbutyric oxime di TMS	15.12	—	16.19	15.14	16.37
Trans-cinnamic mono TMS	15.26	—	16.78	15.21	17.17
3-Methyladipic di TMS	15.28	15.44	16.40	15.28	16.20
3-Methylcrotonylglycine mono TMS	—	—	—	15.39	17.60
Tiglyglycine mono TMS	15.41	15.79	17.93	15.49	17.69
4-Phenylbutyric mono TMS	15.10	—	16.40	15.21§	—
Furan-5-hydroxymethyl-2-Carboxylic di TMS	—	15.61	—	15.28§	—
Tiglylglycine di TMS	—	—	—	15.49	16.76
Undecanoic mono TMS	15.49	—	16.10	15.49	16.04
Erythronic tetra TMS	—	15.67	—	15.49§	—
Threonic tetra TMS	—	15.83	—	15.63§	—
2-Hydroxyphenylacetic di TMS	—	15.80	—	15.59	16.90
n-Valerylglycine di TMS	15.60	—	16.81	15.59	16.67
2-Hydroxyglutaric tri TMS	15.76	15.88	16.66	15.77	15.85
3-Methylcrotonylglycine di TMS	15.60	—	16.70	15.63	16.97
Lauric mono TMS	16.47	—	17.06	16.51	16.97
3-Hydroxyphenylacetic di TMS	15.96	16.19	17.22	15.98	17.33
Pimelic di TMS	15.92	16.10	17.12	15.91	16.93
Phenyllactic di TMS	15.83	16.00	16.89	15.80	16.88
2-Oxoglutaric-EO di TMS	—	—	—	15.98§	—
4-Hydroxybenzoic di TMS	16.22	16.37	17.43	16.22	17.30

Table 3
(Continued)

Compound	Retention index methylene units				
	DB-1*	Ultra-2*	DB-1701*	OV-1‡	OV-17‡
3-Hydroxy-3-methylglutaric tri TMS	16.06	16.17	16.85	16.12	16.48
Cis-oxalacetic oxime di TMS	15.53	—	16.21	15.28	16.14
2-Oxoglutaric oxime tri TMS	16.27	16.45	17.20	16.33	17.06
4-Hydroxyphenylacetic di TMS	16.27	16.50	17.57	16.25	17.66
3-Phenylpyruvic-EO mono TMS	—	—	—	16.14§	—
3-Phenylpyruvic oxime di TMS	16.30	16.62	17.43	16.32	17.81
Furan-2,5-dicaroxylic di TMS	—	16.55	—	16.26	17.96
Tartaric tetra TMS	16.55	16.65	17.21	16.47§	—
Furan-2-carboxylglycine di TMS	—	16.64	—	16.17	18.97
Suberic di TMS	16.87	17.10	18.10	16.91	17.94
2-Oxoadipic-EO di TMS	—	—	—	17.12§	—
2-Oxoadipic oxime tri TMS	17.07	—	18.08	17.21	17.98
Quinolinic di TMS	—	17.45	—	17.28§	—
Homogentisic tri TMS	18.34	—	19.47	17.28§	—
Aconitic tri TMS	17.44	17.64	18.71	17.53	18.41
4-Hydroxy-3-methoxybenzoic di TMs	17.54	17.76	18.94	17.54	19.08
Orotic tri TMS	17.44	—	18.47	17.49	18.59
Tridecanoic mono TMS	17.48	—	18.09	17.49	17.99
Homovanillic di TMS	17.59	17.86	19.12	17.58	19.32
Gentisic tri TMS	17.80	—	18.82	17.93§	—
Azelaic di TMS	17.82	18.12	19.08	17.89	18.88
Hippuric mono TMS	18.05	18.46	20.34	17.96	21.10
Hippuric di TMS	17.92	18.22	19.80	17.85	20.04
Ribonic penta TMS	—	17.99	—	18.29§	—
Arabinonic penta TMS	—	18.12	—	18.38§	—
Tetradecanoic mono TMS	18.40	—	19.03	18.45	18.96
Citric tetra TMS	18.35	18.53	19.17	18.41	18.69
Isocitric tetra TMS	18.35	—	19.27	18.41	18.86
Methylcitric tetra TMS	18.58	18.74	19.25	18.66	18.92
3,4-Dihydroxymandelic tetra TMS	19.36	—	20.19	19.56§	—
Vanillylmandelic tri TMS	—	19.05	—	18.83§	—
3-Indoleacetic di TMS	18.80	19.79	22.42	19.74	23.61
Sebacic di TMS	18.84	—	20.09	18.90	19.95
4-Hydroxyphenyllactic tri TMS	19.06	19.21	20.10	19.09	19.93
4-Hydroxyphenylpyruvic-EO di TMS	—	—	—	19.38§	—
4-Hydroxyphenylpyruvic oxime tri TMS	19.34	19.60	20.44	—	—
Pentadecanoic mono TMS	19.43	—	20.04	19.44	19.95
2-Hydroxyhippuric tri TMS	19.52	—	21.03	19.54	21.01
Palmitoleic mono TMS	20.16	—	20.92	20.31§	—
Palmitic mono TMS	20.40	20.50	21.02	20.43	20.90
2-Hydroxyhippuric di TMS	20.50	—	23.26	20.47	23.03
Uric tetra TMS	21.35	21.35	—	21.08§	—
5-Hydroxyindoleacetic tri TMS	21.99	—	24.01	22.00	25.14
Oleic mono TMS	22.08	—	22.85	22.08	22.87
Linoleic mono TMS	22.01	—	22.95	22.00	23.00
Stearic mono TMS	22.39	—	23.07	22.39	22.90
4-Hydroxyhippuric tri TMS	—	22.39	—	21.25	22.78

* Retention indices on fused silica open tubular column with bonded methylsilicone (DB-1) or 85% methyl, 7% cyanopropyl, 7% phenyl, 1% vinylpolysiloxane (DB-1701), respectively, ref. 119.
† Retention indices on fused silica open tubular column with 5% phenylmethyl silicone (Ultra-2), ref. 120.
‡ Retention indices on column packed with 10% OV-1 and 10% OV-17, respectively, ref. 99.
§ Retention indices of oxo acid ethoximes (EO) and of other acids on % OV-1 packed column, ref. 100.

libraries specializing in organic acids or metabolites of medicaments may involve 300–1000 mass spectra so that the search is faster. The application of a collection of spectra "Mass Spectra of Compounds of Biological Interest" compiled by Markey *et al.* [101] is suitable when identifying an unknown substance. Among the computer programs for identification of organic acid profiles in biological fluids, CLEANUP, TIMSEK, SEARCH and HISLIB [102, 103] may be cited as examples. The programs perform the following operations:

a. detection of mass spectra of substances in GC/MS data;
b. resolution of components insufficiently separated by GC;
c. subtraction of the background caused by column bleeding;
d. calculation of relative retention indices (RRI) and relative concentrations of components of the analyzed mixtures;
e. comparison of mass spectra of components with library spectra. For comparison, mass spectra of standards with the same RRI as the component of interest within ±10 RRI units are expected;
f. comparison of the obtained GC/MS profile with profiles stored in the computer memory, calculation of statistics comparing concentrations and retention characteristics of the substances.

This sequence of computer programs was used for diagnosis of inherited metabolism disorders and for comparison of extraction methods used for isolation of organic acids from body fluids [104].

Among the computer programs for quantification and identification of organic acids and metabolites, the following programs should be mentioned: MSSMET described in several papers [105–107], for (1) non-parametric pattern recognition and features extraction techniques used for comparison of metabolite and volatile matter profiles in biological fluids [108]; (2) identification of anomalies in multi-component mixtures [109] and self-training, interpretative and retrieval system (STIRS) for mass spectra [110].

In many cases of analyses of organic acids in biological fluids and in diagnostics of inherited errors of metabolism it is not necessary to use computer evaluation of results of GC/MS analyses as the changes of organic acid profiles are readily apparent and can be characterized by simpler techniques. On the other hand, a suitable computer system with specialized software will make screening of metabolic disorders easier when many patients are involved and enable identification of less marked but, nevertheless, important changes in excretion of organic acids.

Conclusions

This paper is aimed at reviewing the current state in methodology of analysis of organic acids in biological fluids. However, technology in this field of biochemical analysis is rapidly developing as in other areas of biochemical analysis. Developments include new methods for pre-cleaning of samples and isolation of substances to be determined (e.g. application of ready-to-use columns for extraction) on the one hand and marked developments in analytical instrumentation on the other. Future developments may lead to elimination of the first steps of the determination method (pre-cleaning, isolation) by using high efficiency methods for separation, e.g. combination MS/MS. Increasing separation possibilities of gas chromatography enable separation and quantification of substances unknown till now. In the surveys from 1981–1983 (e.g. 111), separations were nearly always performed in packed columns; now the majority of

authors use high efficiency capillary columns. Simultaneously, mass spectrometers of new design have been introduced into the market enabling very fast scanning (up to 4000 amu s^{-1}) and are suitable for connection with capillary chromatography without deterioration of the separation efficiency. For smaller laboratories simple mass spectrometers have become available with quadrupole filter (mass selective detector, MSD) or with the so-called ion trap detector, ITD, [112]. These find broad application and combination of these small mass spectrometers in the assembly MS/MS is also reported. Development continues, also in other fields of analytical instrumentation resulting in instruments giving quite new possibilities for analysis of components of biological fluids, e.g. modern GC/FTIR instruments enabling capture of column eluate on a cooled plate for subsequent analysis, high-sensitive NMR instruments, FTMS instruments with high resolution for simplifying the identification of substances are available in the market. It is needless to say that developments are favoured also by the development of computer software.

It may be presumed that this development will lead to speeding up the analysis. This will enable new screening methods to be introduced. Identification of substances will be made easier; this will lead to identification of new, unknown till now components of biological fluids and, probably, to extensions of analysis to biological materials used rarely till now, e.g. tissue homogenates.

This progress could lead to deeper understanding of metabolic processes in the organism, and introduction of new therapeutic processes.

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