CAPILLARY GAS CHROMATOGRAPHICAL ANALYSIS AND MASS SPECTROMETRIC IDENTIFICATION OF POLYCYCLIC AROMATIC HYDROCARBON METABOLITES FROM BIOLOGICAL MATERIALS

Jürgen Jacob* and Gernot Grimmer
Biochemisches Institut für Umweltcarcinogene
2070 Ahrensburg-Grosshansdorf, FRG

SUMMARY

Supplementary to HPLC, capillary gas chromatography (CGC) can be used in combination with mass spectrometry (MS) for the characterization and identification of polycyclic aromatic hydrocarbon (PAH) metabolites from various biological systems. The main advantage of this technique can be seen in (a) the high resolution of CGC which is superior to HPLC, (b) the predictability of response factors according to the dependence of the FID-signals on the carbon content, and (3) the far-reaching and readily obtainable information on the structure of metabolites by MS.

A disadvantage is that volatility of the metabolites is required and that extremely polar compounds (such as glucuronides, sulfates, etc.) cannot be analyzed by this method. These problems can be partially overcome by derivatizing the metabolites.

INTRODUCTION

During the past decade substantial work on the metabolism of PAH in eukaryotic and procaryotic organisms has been carried out which has improved our understanding of PAH-induced carcinogenesis. Most investigators have used high performance liquid chromatography (HPLC) for analyzing the metabolite profiles of various PAH in different biological systems. Analysis of benzo(a)pyrene metabolism by

means of HPLC has been reviewed by Selkirk /1/ and by Yang *et al.* /2/. For the HPLC separation of critical pairs such as 1- and 3-hydroxy-benzo(a)pyrene multifold chromatography is required. The same holds true for various trans-dihydrodiols of benz(a)anthracene /3/. The primary metabolism of a number of other environmentally relevant and/or carcinogenic PAH has also been studied by HPLC, e.g. fluorene /4/, phenanthrene /5/, fluoranthene /6/, chrysene /7/, 5-methylchrysene /8/, 7,12-dimethylbenz(a)anthracene /9,10/, benzo(c)phenanthrene /11/, cyclopenta(cd)pyrene /12/, cholanthrene /13/, and 3-methylcholanthrene /14/, benzo(e)pyrene /15/, benzo(b)-, -(j)-, and -(k)fluoranthene /16,17/, indeno(1,2,3-cd)pyrene /18,19/, di-benz(a,h)anthracene /20/, dibenzo(a,h)- and dibenzo(a,i)pyrene /21/.

For the analysis of the secondary metabolism of PAH forming water-soluble products such as glucuronides, sulfates, glutathion-, protein-, and DNA-conjugates HPLC is the most used technique. The hydrophilic metabolites of benzo(a)pyrene have been thoroughly investigated by many authors, e.g. Baird *et al.* /22/ and by MacLeod *et al.* /23/.

HPLC was the most commonly available method at the time in those laboratories initially involved in PAH metabolism studies. This may explain why CGC and GC/MS have been neglected for such a long time in this field. To avoid tailing effects and decomposition primary metabolites such as phenols and dihydrodiols have to be converted to acylates, trimethylsilyl or methyl ethers. The elaboration of the derivatization method has delayed the application of the CGC technique to metabolic studies so that only a few attempts have been made /e.g. 24-27/ until recently.

This paper summarizes GC and GC/MS studies of the metabolism of polycyclic aromatic compounds (PAC) such as phenanthrene, pyrene, benz(a)anthracene, chrysene, benzo(e)pyrene, indeno(1,2,3-cd)pyrene, benz(a)- and benz(c)acridine, benzo(b)naphtho(1,2-d)-, -(2,1-d)- and -(2,3-d)thiophene as well as triphenylene(4,5-bcd)thiophene).

METHODOLOGY

The general scheme for isolation and characterization or identification of PAH metabolites requires (a) extraction and clean-up, (b) deriva-

tization by conversion to trimethylsilyl ethers, (c) gas chromatographic separation and (d) mass spectrometric analysis. For special cases, when, for instance, only the analysis of phenols from urine samples is demanded, an alternative method can be recommended during which phenols are converted into phenolmethyl ethers by diazomethane treatment. The latter can be analyzed readily by GC/MS (see under f).

a. Clean-up by chromatography on Sephadex LH 20

Prior to GC/MS, PAH metabolites together with the unconverted PAH substrates had to be enriched from biological systems (tissues, microsomes, cell or tissue cultures) by extraction preferably with ethyl acetate under slightly acidic conditions ($\text{pH} \leq 3$) in order to extract phenols. After washing this extract with water, it was cautiously evaporated almost to dryness and then chromatographed on Sephadex LH 20 with isopropanol as elutant to get rid of non-aromatic compounds and most of the laboratory contaminants. The elution volumes for a number of PAH, azaarenes, nitro-PAH, and thiaarenes are listed in Table 1. The fraction eluted before the unconverted PAH substrate is discarded. *Trans*-dihydrodiols and tetrols require about 10% and phenols about 30% larger elution volumes than the base PAH. In order not to lose any of the metabolites possibly formed, the fractions are generously dimensioned (10-20 mL prior to when the substrate appears and at least 100 mL, or preferably 150 mL, after it has disappeared from the column).

b. Preparation of the sample for CGC (derivatization)

The Sephadex LH 20 column eluate is evaporated and the residue dissolved in 10 μL toluene. To this solution 8 μL of a mixture of Trisil (Pierce) and N,O,bis-(trimethylsilyl)trifluoroacetamide (BSTFA Pierce) (1:1; v:v) is added. The silylation is complete after 45 min at 45°C. Due to possible decomposition by moisture it is advantageous to carry out the GC analysis immediately after derivatization. However, silylated PAH-metabolites can be stored under nitrogen in the dark at -10°C for at least 5 days without any detectable decomposition.

TABLE 1

Elution volumes of various PAH, azaarenes, nitro-PAH and thiaarenes on a Sephadex LH 20 column (10 g; isopropanol as elutant).

Compound	Elution volume (ml)
PAH	
Anthracene	38- 50
Phenanthrene	38- 50
Fluoranthene	48- 63
Pyrene	48- 63
Benzofluorenes (a+b+c)	51- 63
Benzo(ghi)fluoranthene	56- 71
Cyclopenta(cd)pyrene	60- 78
Benzo(a)anthracene	60- 78
Chrysene	60- 78
Benzofluoranthenes (b+j+k)	72- 90
Benzo(a)pyrene	73- 93
Benzo(e)pyrene	78- 93
Perylene	83-106
Indeno(1,2,3-cd)fluoranthene	84-107
Indeno(1,2,3-cd)pyrene	84-107
Benzo(b)chrysene	84-118
Anthanthrene	88-113
Benzo(ghi)perylene	90-118
Coronene	104-140
Azaarenes	
Acridine	31- 51
Benzo(f)quinoline	35- 55
Benz(a)acridine	43- 67
Benz(c)acridine	50- 77
Carbazol	51- 80
Dibenz(a,j)acridine	59- 90
Benzo(c)carbazol	80-113
Dibenzo(c,g)carbazol	93-140
Nitro-PAH	
1-Nitronaphthalene	50- 63
2-Nitronaphthalene	50- 63
2-Nitrofluorene	52- 69
9-Nitroanthracene	52- 72
3-Nitrofluoranthene	57- 82
1-Nitropyrene	59- 85
Thiaarenes	
Benzo(b)naphtho(1,2-d)thiophene	53- 78
Benzo(b)naphtho(2,1-d)thiophene	48- 68
Benzo(b)naphtho(2,3-d)thiophene	53- 76
Triphenyleno(4,5-bcd)thiophene	68- 95

c. Capillary gas chromatography (CGC)

For the GC analysis, 25-50 m (0.2-0.4 mm inner diameter) capillaries coated with dimethylpolysiloxane (e.g. SE-30, OV 101, OV 1), 95% dimethyl-/ 5% diphenylpolysiloxane (e.g. SE-52, SE-54) and comparable materials or chemically bound fused silica columns (e.g. CP sil 5 CB) are used. It is important to use all glass systems to avoid decomposition of TMS-ethers during GC, especially in the injection port. On-column and cold injection are the recommended inlet systems. Samples may be injected for instance at 100°C column temperature held for 5 or 10 min at that temperature to let the solvent and the excess of silylation reagent pass through the column. Thereafter a temperature program of 100-260°C with 2°C/min can be chosen. Sometimes it is advantageous to start with a rapid program of 30°C/min from 100-160°C followed by a flat temperature program of 1.5°C or 2°C/min from 160-270°C. Helium is used as a carrier gas with flow rates of 20-25 cm/sec (equal to 2-3 mL/min). To minimize peak tailing a post-column acceleration with argon between the column outlet and the detector system (FID) is recommended. To avoid detector coating by the excess of the silylation reagent, which may decrease the sensitivity, the electrode is removed for the first 5-10 minutes during which no metabolite peaks are expected. About 0.1 ng is the limit of detection for a well adjusted GC system.

d. Mass spectrometry (MS)

Double focussing MS is advantageous but not obligatory. For optimum results the same GC equipment as mentioned under c. is required. To avoid an unnecessary burden of the ion source by the solvent and the silylation reagent of the sample, the interface between the GC and MS is not connected before 5-7 minutes after injection to blend out these components. Thus, a large sample of a total volume of 5-10 µL can be used. Mass spectra are mostly recorded at 70-80 eV to obtain intense fragmentation. Parent peak spectrometry, however, is carried out at 20 eV.

e. Reference materials

Various PAH, azaarenes and thiaarenes can be obtained from the Commission of the European Communities, Community Bureau of Reference — BCR, 200 Rue de la Loi, B-1049 Brussels, in high grade purity (> 99.0%) as reference standards. From this collection indeno(1,2,3-cd)fluoranthene has been found to be an excellent internal standard; in the low boiling range benzo(c)phenanthrene or 3,6-dimethylphenanthrene can be used as internal standards. However, there is a lack of PAH-metabolite standard materials and only a few PAH phenols are commercially available. A larger collection of PAH-metabolites can be obtained in milligram amounts from the NCI Chemical Repository, Bethesda, USA.

f. Identification of phenols as phenolmethyl ethers (methoxy-PAH)

For determination of PAH-metabolites in urine an alternative method can be used which allows the quantitative GC analysis of phenols after methylation in form of the phenol methyl ethers (methoxy-PAH).

RESULTS AND APPLICATIONS

a. Capillary gas chromatography

CGC of trimethylsilyl ethers of PAH metabolites has been successfully applied in the separation of isomers. In case of phenanthrene the various phenol and dihydrodiol TMS-ethers exhibit differences in retention times relative to the parent PAH (RRT) which allow an almost complete separation from each other. The RRT-values of the phenanthrene metabolites are listed in Table 2 and a gas-chromatogram of a separation of some of them is presented in Figure 1.

It seems not to be accidental that the 4-isomer (followed by 9-isomer) exhibits the shortest RRT; so far as is known, this holds true for several PAH, the OTMS-substituent of which is located in the bay-region. This effect may be due to the more compact structure in

Fig. 1: CGC-separation of a synthetic mixture of phenanthrene metabolites after silylation (CGC conditions: Gas chromatograph Perkin-Elmer 8320 on column injection, column SE 54 chemical bound, 25 m x 0.32 mm, 0.25 μ m film thickness, carrier gas helium; 2 ml/min, FID-temperature 280°C). Note: According to different GC-conditions the elution sequence of the metabolites differs from that given in Table 2.

TABLE 2

GC retention times of various phenanthrene metabolites (relative to phenanthrene) after silylation (25 m x 0.4 mm glass capillary column coated with CP sil 5 (0.8 μ m film thickness) /28/

Compound	RRT
Phenanthrene	1.000
1-Hydroxyphenanthrene	1.605
2-Hydroxyphenanthrene	1.666
3-Hydroxyphenanthrene	1.613
4-Hydroxyphenanthrene	1.497
9-Hydroxyphenanthrene	1.585
<i>trans</i> -1,2-dihydroxy-1,2-dihydrophenanthrene	1.832
<i>trans</i> -3,4-dihydroxy-3,4-dihydrophenanthrene	1.526
<i>trans</i> -9,10-dihydroxy-9,10-dihydrophenanthrene	1.518
<i>cis</i> -9,10-dihydroxy-9,10-dihydrophenanthrene	1.590

comparison to the more stretched molecular shape to be expected, e.g. for the 2-phenanthrol TMS-ether. As a consequence, among the dihydrodiol-TMS-ethers the *trans*-1,2-dihydrodiol shows the greatest RRT. However, exceptions to this rule have also been observed. *Cis*-isomers of the dihydrodiols generally exhibit larger retention times than *trans*-isomers.

The aforementioned findings have been confirmed with the phenols and dihydrodiols of benz(a)anthracene as can be seen from Table 3. The TMS-ethers of all these metabolites can be separated by CGC as shown in Figure 2.

TABLE 3

GC retention times of various benz(a)anthracene metabolites (relative to benz(a)anthracene) after silylation (25 m x 0.3 mm glass capillary column coated with CP sil 5 (0.8 μ m film thickness) /29/

Compound	RRT
Benz(a)anthracene	1.000
1-Hydroxybenz(a)anthracene	1.171
2-Hydroxybenz(a)anthracene	1.228
3-Hydroxybenz(a)anthracene	1.258
4-Hydroxybenz(a)anthracene	1.240
5-Hydroxybenz(a)anthracene	1.218
8-Hydroxybenz(a)anthracene	1.215
9-Hydroxybenz(a)anthracene	1.259
10-Hydroxybenz(a)anthracene	1.253
11-Hydroxybenz(a)anthracene	1.184
<i>trans</i> -1,2-Dihydroxy-1,2-dihydrobenz(a)anthracene	1.127
<i>trans</i> -3,4-Dihydroxy-3,4-dihydrobenz(a)anthracene	1.312
<i>trans</i> -5,6-Dihydroxy-5,6-dihydrobenz(a)anthracene	1.100
<i>cis</i> -5,6-Dihydroxy-5,6-dihydrobenz(a)anthracene	1.110
<i>trans</i> -8,9-Dihydroxy-8,9-dihydrobenz(a)anthracene	1.290
<i>trans</i> -10,11-Dihydroxy-10,11-dihydrobenz(a)anthracene	1.260

The RRT for chrysene metabolites are summarized in Table 4.

In Figure 3, a CGC separation of some possible primary chrysene metabolites is shown.

CGC data are also available for some metabolites of pyrene, benzo(e)pyrene, and indeno(1,2,3-cd)pyrene; they are listed in Table 5.

Fig. 2: CGC-separation of a mixture of benz(a)anthracene and its metabolites after silylation (CGC-conditions are given in Table 3).

Fig. 3: CGC-separation of a mixture of chrysene and its metabolites after silylation (CGC-conditions are given in Table 4).

TABLE 4

GC retention times of various chrysene metabolites (relative to chrysene) after silylation (25 m x 0.3 mm glass capillary column coated with CP sil 5, 0.8 μ m film thickness) /30/.

Compound	RRT
Chrysene	1.000
1-Hydroxychrysene	1.192
2-Hydroxychrysene	1.211
3-Hydroxychrysene	1.188
4-Hydroxychrysene	1.133
6-Hydroxychrysene	1.143
<i>trans</i> -1,2-Dihydroxy-1,2-dihydrochrysene	1.261
<i>trans</i> -3,4-Dihydroxy-3,4-dihydrochrysene	1.108
<i>trans</i> -5,6-Dihydroxy-5,6-dihydrochrysene	0.988

Figure 4 shows that the separation of some isomers may be difficult in the case of the benzo(e)pyrene phenol TMS-ethers (e.g. 1-; 4- and 10-isomer).

The relative retention time of a number of benzo(a)pyrene metabolites can be calculated from the data reported by Takahashi *et al.* /25/. They are presented in Table 6. The bay-region phenols (10- and 11-hydroxybenzo(a)pyrene) were found to have the lowest values, whereas the 8-hydroxybenzo(a)pyrene exhibits the highest RRT. Slight differences in the ranking of RRTs were found when two different stationary phases were applied (Dexsil-300 and OV-1) /25/. Although *cis*-dihydrodiol-TMS-ethers generally exhibit greater RRT than the

Fig. 4: CGC-separation of a mixture of benzo(e)pyrene and its metabolites after silylation (CGC-conditions are given in Table 5).

TABLE 5

Relative GC retention times of some pyrene, benzo(e)pyrene, and indeno(1,2,3-cd)pyrene metabolites after silylation (25 m x 0.4 mm glass capillary column coated with CP sil 5; film thickness 0.8 μ m) /31-34/.

Metabolite	RRT
Pyrene	
1-Hydroxypyrene	1.343
1,6-Dihydroxypyrene	1.612
1,8-Dihydroxypyrene	1.590
<i>trans</i> -4,5-Dihydroxy-4,5-dihydropyrene	1.211
Benzo(e)pyrene	
1-Hydroxybenzo(e)pyrene	1.235
3-Hydroxybenzo(e)pyrene	1.143
4-Hydroxybenzo(e)pyrene	1.235
9-Hydroxybenzo(e)pyrene	1.208
10-Hydroxybenzo(e)pyrene	1.240
<i>trans</i> -4,5-Dihydroxy-4,5-dihydrobenzo(e)pyrene	1.047
<i>trans</i> -9,10-Dihydroxy-9,10-dihydrobenzo(e)pyrene	1.058
Indeno(1,2,3-cd)pyrene	
2-Hydroxy-indeno(1,2,3-cd)pyrene	1.437
6-Hydroxy-indeno(1,2,3-cd)pyrene	1.226
7-Hydroxy-indeno(1,2,3-cd)pyrene	1.291
8-Hydroxy-indeno(1,2,3-cd)pyrene	1.375
<i>trans</i> -1,2-Dihydroxy-1,2-dihydroindeno-(1,2,3-cd)pyrene	1.063

Pyrene

Benzo(e)pyrene

Indeno(1,2,3-cd)pyrene

TABLE 6

GC retention times of various benzo(a)pyrene metabolites (relative to benzo(a)pyrene) after silylation (1 m x 2 mm packed column with 2% OV-1 or 2 m x 2 mm packed column with 6% Dexsil-300 on Chromsorb W, operated at 250°C (OV-1) and 290°C (Dexsil-300)) /25/

Metabolite	Dexsil-300	OV-1
Benzo(a)pyrene		
Benzo(a)pyrene	1.00	1.00
1-Hydroxybenzo(a)pyrene	2.73	2.73
2-Hydroxybenzo(a)pyrene	2.73	3.31
3-Hydroxybenzo(a)pyrene	2.73	3.42
4-Hydroxybenzo(a)pyrene	2.35	2.92
5-Hydroxybenzo(a)pyrene	2.05	2.61
6-Hydroxybenzo(a)pyrene	2.00	2.44
7-Hydroxybenzo(a)pyrene	2.38	2.97
8-Hydroxybenzo(a)pyrene	3.05	3.67
9-Hydroxybenzo(a)pyrene	2.62	3.28
10-Hydroxybenzo(a)pyrene	1.92	2.42
11-Hydroxybenzo(a)pyrene	1.59	2.11
12-Hydroxybenzo(a)pyrene	1.95	2.47
Dihydroxybenzo(a)pyrene		
1,6-Dihydroxybenzo(a)pyrene	4.19	6.11
3,6-Dihydroxybenzo(a)pyrene	3.65	5.58
4,5-Dihydroxybenzo(a)pyrene	2.14	3.58
6,12-Dihydroxybenzo(a)pyrene	2.81	4.69
7,10-Dihydroxybenzo(a)pyrene	3.24	4.94
11,12-Dihydroxybenzo(a)pyrene	1.24	2.08
Dihydrodihydroxybenzo(a)pyrene		
<i>cis</i> -4,5-Dihydroxy-4,5-dihydro-BaP	0.57	1.44
<i>trans</i> -4,5-Dihydroxy-4,5-dihydro-BaP	0.32	1.03
<i>cis</i> -7,8-Dihydroxy-7,8-dihydro-BaP	1.73	3.00
<i>trans</i> -7,8-Dihydroxy-7,8-dihydro-BaP	2.24	3.78
<i>trans</i> -9,10-Dihydroxy-9,10-dihydro-BaP	0.65	1.53
<i>cis</i> -11,12-Dihydroxy-11,12-dihydro-BaP	0.32	1.08
<i>trans</i> -11,12-Dihydroxy-11,12-dihydro-BaP	0.08	0.39

TABLE 6 (Cont.)

7 β , 8 α , 9 β , 10 α -Tetrahydroxy-7,8,9,10-*	1.35	4.17
7 β , 8 α , 9 β , 10 β -Tetrahydroxy-7,8,9,10-*	2.84	7.22
7 β , 8 α , 9 α , 10 α -Tetrahydroxy-7,8,9,10-*	1.70	4.94
7 β , 8 α , 9 α , 10 α -Tetrahydroxy-7,8,9,10-*	2.35	6.13
Benzo(a)pyrene-1,6-quinone	3.89	3.42
Benzo(a)pyrene-3,6-quinone	4.27	3.67
Benzo(a)pyrene-4,5-quinone	4.92	4.03
Benzo(a)pyrene-6,12-quinone	2.97	2.81
Benzo(a)pyrene-7,10-quinone	1.86	1.97
Benzo(a)pyrene-11,12-quinone	4.54	3.47

* tetrahydrobenzo(a)pyrene

corresponding *trans*-isomers, the opposite was reported for the two isomers of the 7,8-benzo(a)pyrenediol /25/.

CGC retention data of some metabolites derived from heterocyclic PAC such as benz(a)- and benz(c)acridine as well as various thiaarenes are presented in Table 7.

It has to be kept in mind that the RRT depend to a great extent on the CGC conditions (temperature, temperature program, column impregnation) so that even the sequence of various metabolites may occasionally differ under varying conditions.

Since the response of FID generally used in CGC strongly depends linearly on the carbon content of the molecule, identical intensities of the signals are expected and observed for a certain class of compound (e.g. phenol TMS-ethers or dihydrodiol TMS-ethers). An example shows that the relative responses increase with the degree of oxygenation for a

TABLE 7

Relative GC retention times of metabolites of some heterocyclic PAC after silylation (except the thiaarene metabolites) (25 m x 0.37 mm glass capillary column coated with CP sil 5; film thickness 0.8 μm) /34-37/

Metabolite	RRT
Benz(a)acridine	1.000
<i>trans</i> -3,4-Dihydroxy-3,4-dihydrobenz(a)acridine	1.395
<i>trans</i> -5,6-Dihydroxy-5,6-dihydrobenz(a)acridine	1.108
Benz(c)acridine	1.000
<i>trans</i> -3,4-Dihydroxy-3,4-dihydrobenz(c)acridine	1.444
<i>trans</i> -5,6-Dihydroxy-5,6-dihydrobenz(c)acridine	1.155
3,4,5,6-Tetrahydroxy-3,4,5,6-tetrahydro-BcAc	1.861
Benzo(b)naphtho(1,2-d)thiophene	1.000
Benzo(b)naphtho(1,2-d)thiophene-7,7-dioxide	1.380
Benzo(b)naphtho(2,1-d)thiophene	1.000
Benzo(b)naphtho(2,1-d)thiophene-11,11-dioxide	1.360
Benzo(b)naphtho(2,3-d)thiophene	1.000
Benzo(b)naphtho(2,3-d)thiophene-5,5-dioxide	1.360
Benzo(b)naphtho(2,3-d)thiophene-6,11-quinone	1.120
Triphenyleno(4,5-bcd)thiophene	1.000
Triphenyleno(4,5-bcd)thiophene-4,4-dioxide	1.290

Benz(a)acridine

Benz(c)acridine

Benzo(b)naphtho(1,2-d)thiophene

Benzo(b)naphtho(2,1-d)thiophene

Benzo(b)naphtho-(2,3-d)thiophene

Triphenyleno(4,5-bcd)thiophene

distinct PAH: in case of benz(a)anthracene the relative carbon content is 94.7%; if the FID-signal for the base PAH is set to 100%, the relative response for a phenol TMS-ether derived from a certain absolute amount of this PAH (e.g. 1 nanomole = 228 ng) should be 1.17 times this signal, since the molecular weight, and hence the carbon number per molecule, increases from 228 in benz(a) anthracene ($C_{18} \cong 216$) to 244 in case of the phenol and 316 in case of the phenol TMS-ether ($C_{21} \cong 252$). Accordingly, for the FID-signal a factor of $252/216 = 1.17$ is to be expected which, roughly, is what is found. In the case of dihydrodiol TMS-ethers this factor rises to 1.33. In Table 8, theoretical response factors for some types of metabolites derived from a number of PAH are listed.

Recoveries of phenols and dihydrodiols for the complete method including clean-up and CGC have been found to be about 90% with coefficients of variation between 5-8%. GC recoveries (response) for the set of methoxyphenanthrenes have been measured recently [38] and found to lie between 91-106% with coefficients of variation of 2.4-4.1% (Table 9).

Relative retention times for a number of methoxy-PAH are summarized in Table 10.

Although the RRT values of methoxy-PAH do not always appear in the same order as those of the TMS-ethers, the bay-region substituted methoxy-PAH generally exhibit the shortest retention times.

b. Mass spectrometry (MS)

Apart from the molecular weight of the metabolites, many items of information on the molecule may be obtained by MS. In most cases, prominent molecular ions are observed, mainly base peaks in the case of the PAH phenol TMS-ethers (see Table 11) and most of the quinones. They are smaller in case of the TMS-ethers of dihydrodiols, triols and tetrols.

For phenols, the ratio M-15/M-31 is an indication of the location of the substituent. Sometimes low ratios are found for positions where a 5- or 6-membered cyclic ion can be formed by elimination of ($2 CH_3 + H$) /28,33/ as in the case of 3-hydroxy-benzo(e)pyrene-TMS-ether ((a), Figure 5) or 4-hydroxy-phenanthrene-TMS-ether ((b), Figure 5),

TABLE 8

GC response factors for some types of metabolites derived from various PAH, when detected as methyl- or TMS-ethers

Hydrocarbon	Phenol methyl-ether	TMS-ether	Dihydrodio- TMS-ether	Triol TMS-ether	Tetro-
Phenanthrene/Anthracene	1.00	1.07	1.21	1.43	1.64
Pyrene/Fluoranthene	1.00	1.06	1.19	1.38	1.56
Benz(a)anthracene/Chrysene/Benzo- (c)phenanthrene/Triphenylene	1.00	1.06	1.17	1.33	1.50
Benzo(a)pyrene/Benzo(e)pyrene/	1.00	1.05	1.15	1.30	1.45
Benzo(a)fluoranthene	1.00	1.05	1.14	1.27	1.41
Indeno(1,2,3-cd)pyrene					1.55

TABLE 9

GC recoveries and their variation coefficients of various methoxyphenanthrenes (n=6)

Compound	Recoveries						mean V (%)	
1-Methoxy-PHE	104	14	105	99	103	100	101.8	2.44
2-Methoxy-PHE	96	95	100	97	105	102	99.2	3.90
3-Methoxy-PHE	98	90	95	95	95	98	95.2	3.08
4-Methoxy-PHE	101	102	98	100	106	95	100.3	3.71
9-Methoxy-PHE	92	98	90	90	87	90	91.2	4.07

TABLE 10

GC retention times of various methoxy-PAH (related to the base PAH (25 m x 0.32 mm) Nordibond (chemical bound) SE-54, 0.25 μ m film thickness, fused silica; 110-185°C with 25°C/min, then 185°C - 280°C with 2°C/min

Compound	RRT
Phenanthrene	1.000
1-Methoxyphenanthrene	1.585
2-Methoxyphenanthrene	1.604
3-Methoxyphenanthrene	1.548
4-Methoxyphenanthrene	1.503
9-Methoxyphenanthrene	1.558
Pyrene	
1-Methoxypyrene	1.693
Chrysene	1.000
1-Methoxychrysene	1.393
2-Methoxychrysene	1.404
3-Methoxychrysene	1.370
4-Methoxychrysene	1.341
6-Methoxychrysene	1.330
Benzo(a)pyrene	1.000
3-Methoxybenzo(a)pyrene	1.271
9-Methoxybenzo(a)pyrene	1.253

TABLE 11

Main mass spectrometric fragments and their relative intensities of various PAH phenol TMS-ethers (recorded at 80 eV)

Metabolite	Relative intensities (%)				
	M	(M-15)	(M-31)	m/z 73	M-15/M-31
1-OTMS-PYR*	100	29	14	72	2.1
1,6-OTMS-PYR	100	13	7	83	1.9
1,8-OTMS-PYR	100	10	4	59	2.5
1-OTMS-BeP*	10	23	21	25	1.1
3-OTMS-BeP	100	19	26	19	0.7
4-OTMS-BeP	100	19	17	21	1.1
9-OTMS-BeP	100	22	17	17	1.3
10-OTMS-BeP	100	21	3	17	7.0
2-OTMS-INP*	100	31	44	90	0.7
6-OTMS-INP	100	24	19	45	1.3
7-OTMS-INP	100	28	39	28	0.7
8-OTMS-INP	100	21	7	10	3.0
9-OTMS-INP	100	50	11	35	4.5
1-OTMS-PHE*	100	83	27	72	3.1
2-OTMS-PHE	100	94	9	22	10.4
3-OTMS-PHE	100	77	7	22	11.0
4-OTMS-PHE	100	43	47	45	0.9
9-OTMS-PHE	100	77	23	53	3.3
1-OTMS-BaA*	100	32	46	24	0.7
2-OTMS-BaA	100	47	10	29	4.7
3-OTMS-BaA	100	43	9	31	4.8
4-OTMS-BaA	100	42	32	38	1.3
5-OTMS-BaA	100	43	33	46	1.3
8-OTMS-BaA	100	41	26	42	1.6
9-OTMS-BaA	100	62	11	30	5.5
10-OTMS-BaA	100	59	9	34	6.6
11-OTMS-BaA	100	47	34	29	1.4
1-OTMS-CHR*	100	42	17	43	2.5
2-OTMS-CHR	100	52	22	90	2.4
3-OTMS-CHR	100	40	15	55	2.7
4-OTMS-CHR	100	50	46	55	1.1
6-OTMS-CHR	100	48	16	80	3.0

*PYR: pyrene; BeP: benzo(e)pyrene; INP: indeno(1,2,3-cd)pyrene; PHE: phenanthrene; BaA: benz(a)anthracene; CHR: chrysene.

whereas high ratios are found in components with substituents in distal positions such as 2-hydroxy-phenanthrene-TMS-ether ((c), Figure 5) or 10-hydroxy-benzo(e)pyrene-TMS-ether ((d), Figure 5).

In dihydrodiol-TMS-ethers, the base peak is m/z 73 ($^+Si(CH_3)_3$), 147 (tentatively $(CH_3)_2^+Si-O-Si(CH_3)_3$, or 191 ($^+CH(OTMS)_2$). Apart from these, intense fragments (M-15), (M-89), (M-90), and less intense fragments (M-103) and (M-178), can be observed. The intensity of the parent peak is between a few and up to 50% and more, but never becomes the base peak. The ratio m/z 147/191 is indicative of the position of the vicinal dihydroxy substitution. The ratio is $\gg 1$ for K-region dihydrodiol TMS-ethers and < 1 (seldom ≈ 1) in the case of non-K-region dihydrodiol-TMS-ethers. This might be a substantial help for their identification. In Table 12 the relative intensities of some fragments of various PAH-dihydrodiol-TMS-ethers are collected.

Dihydrodiolphenol-TMS-ether (triol-TMS-ether) behaves similarly to dihydrodiol derivatives. The base peak is found among m/z 73, 147, 191. Again a ratio m/z 147/ m/z 191 > 1 indicates a diol TMS- "partial structure" to be present in the K-region, whereas a ratio < 1 indicates its absence in this position. Some of these spectrometric data on PAH-triol-TMS-ether are presented in Table 13.

Less intense parent peaks are observed in the mass spectra of PAH tetraol-TMS-ethers. Table 14 presents some MS data for six different individuals. From these it seems that elimination of a fragment with mass 204 which may be $(CH_3)_3Si-O-HC=CH-O-Si(CH_3)_3$ is preferred in stereo isomers having two quasi-equatorial or quasi-axial adjacent OTMS-groups since the fragment (M-204) is more intense in the 7β , 8α , 9β , 10β -, the 7β , 8α , 9α , 10β - and the 7β , 8α , 9α , 10α - than in the 7β , 8α , 9β , 10α -isomer of the four benzo(a)pyrene tetraols in Table 14, the latter of which possess alternating OTMS-groups.

Quinones can readily be recognized from the fragments (M-28) and (M-56) deriving from CO-eliminations. Moreover, it seems that ortho-quinones can be distinguished from para-quinones by the ratio of $M/(M-28)$ which is > 1 in the latter and < 1 in ortho-quinones (see Table 15).

Sulfoxides and sulfones, the main metabolites of thiaarenes, are mass spectrometrically characterized by a series of key ions, e.g. (M-29), (M-32), (M-45), (M-48), (M-66), and (M-77) corresponding to

Fig. 5: Mass spectrometric behaviour of some phenol TMS-ethers.

TABLE 12

Main mass spectrometric fragments and their relative intensities of various PAH-dihydrodiol-TMS-ethers (recorded at 80 eV)

Meta bolite	M	M-15	M-90	M-103	M-178	Relative intensities (%)			m/z 73	147/191	References
						m/z 191	m/z 147	m/z 73			
<i>tr</i> -1,2-di-OTMS-1,2-dihydro-FHE*	7	18	10	6	20	100	25	98	<1	(28)	
<i>tr</i> -9,10- " 9,10 "	17	22	21	7	14	4	100	65	≥1	(34)	
<i>tr</i> -4,5-di-OTMS-4,5-dihydro-PYR*	15	12	53	4	25	1	72	100	≥1	(31)	
<i>tr</i> -1,2-di-OTMS-1,2-dihydro-llaA*	10	2	25	10	15	100	25	75	<1	(29)	
<i>tr</i> -3,4- " 3,4 "	1	1	8	11	7	75	10	100	<1	(29)	
<i>tr</i> -5,6- " 5,6 "	30	15	33	7	22	6	75	100	≥1	(29)	
<i>cis</i> -5,6- " 5,6 "	32	21	24	7	20	5	100	84	≥1	(29)	
<i>tr</i> -8,9- " 8,9 "	10	3	11	25	11	67	23	100	<1	(29)	
<i>tr</i> -10,11 " 10,11 "	6	2	10	14	7	45	20	100	<1	(29)	
<i>tr</i> -3,4-di-OTMS-3,4-dihydro-BaAc*	3	1	6	6	6	76	27	100	<1	(29)	
<i>tr</i> -3,4-di-OTMS-3,4-dihydro BzAc*	11	3	100	5	5	5	5	100	1	(34)	
<i>tr</i> 1,2-di-OTMS-1,2-dihydro-CHR*	27	9	21	30	27	100	60	75	<1	(30)	
<i>tr</i> 3,4 " 3,4 "	16	9	16	28	14	100	27	100	<1	(30)	
<i>tr</i> 5,6 " 5,6 "	75	21	81	30	75	45	100	100	>1	(30)	

TABLE 12 (Cont.)

<i>tr</i> -4,5-di-OTMS-4,5-dihydro-BeP*	20	11	33	9	18	3	100	31	≥ 1	(33)
<i>tr</i> 9,10 " 9,10 "	22	4	14	17	16	106	11	60	< 1	(33)
<i>tr</i> -4,5-di-OTMS-4,5-dihydro-BaP*	70	21	100	†	9	†	82	33	†	(25)
<i>cis</i> -4,5- " 4,5 "	73	31	77	†	6	†	100	29	†	(25)
<i>tr</i> -7,8 " 7,8 "	21	†	13	42	†	100	†	79	< 1	(25)
<i>cis</i> -7,8 " 7,8 "	18	†	17	44	†	100	14	78	< 1	(25)
<i>tr</i> -9,10 " 9,10 "	42	†	21	31	†	87	13	100	< 1	(25)
<i>tr</i> -11,12 " 11,12 "	83	9	100	†	13	†	40	45	†	(25)
<i>cis</i> -11,12 " 11,12 "	100	16	94	†	8	†	50	15	†	(25)
<i>tr</i> -1,2-di-OTMS-1,2-dihydro-INP*	50	11	72	1	16	1	66	100	≥ 1	(34)

*PHE: phenanthrene; PYR: pyrene; BaA: benz(a)anthracene; BaAc: benz(a)acridine; BcAc: benz(c)acridine; CHR: chrysene; BeP: benzo(e)pyrene; BaP: benzo(a)pyrene; INP: indeno(1,2,3-cd)pyrene.

†No data available.

TABLE 13

Main mass spectrometric fragments and their relative intensities of some PAH-triol TMS-ether (recorded at 80 eV)

Metabolite	M	M-15	M-90	Relative intensities (%)				m/z 147	m/z 73	147/191	References
				M-178	m/z 191	m/z 147	m/z 73				
1,4,5-Tr-OTMS-4,5-dihydro-PYR	15	13	20	7	1	30	100	≥ 1			(31)
1,2,3-Tr-OTMS-1,2-dihydro-BaA	13	7	10	5	38	20	100	< 1			(29)
8,9,10-Tr-OTMS-8,9-dihydro-BaA	8	2	11	6	69	23	100	< 1			(34)
1,2,3-Tr-OTMS-1,2-dihydro-CHR	3	2	1	2	61	12	100	< 1			(34)
1,2,3-Tr-OTMS-1,2-dihydro-CHR	23	9	27	9	100	17	62	< 1			(34)
7,8,9-Tr-OTMS-7,8-dihydro-BaP	68	-	11	-	100	15	64	< 1			(25)

TABLE 14

Mass in mass spectrometric fragments and their relative intensities of some PAH tetraol TMS ethers (recorded at 80 eV)

Metabolite	Relative intensities (%)							References
	M-15	M-90	M-178	M-204	m/z 191	m/z 147	m/z 73	
1,2,3,4-Tetra-OTMS-1,2,3,4-tetrahydro-BaA*	5	1	4	3	100	31	100	(29)
4,5,9,10-Tetra-OTMS-4,5,9,10-tetrahydro-BaP**	4	1	2	3	<1	79	100	(33)
7β, 8α, 9β, 10α-Tetra-OTMS-7,8,9,10-tetrahydro-BaP	—	—	45	—	37	—	33	(25)
7β, 8α, 9β, 10β-Tetra-OTMS-7,8,9,10-tetrahydro-BaP	13	—	—	—	100	—	—	(25)
7β, 8α, 9α, 10β-Tetra-OTMS-7,8,9,10-tetrahydro-BaP	6	—	8	—	80	—	—	(25)
7β, 8α, 9α, 10α-Tetra-OTMS-7,8,9,10-tetrahydro-BaP	5	—	—	—	100	—	—	(25)

*Mixture of stereoisomers

**Configuration unknown

TABLE 15

Main mass spectrometric fragments and their relative intensities of some PAH quinones (recorded at 80 eV)

Metabolite	Relative intensities (%)			
	M	M-28	M-56	References
Acenaphthene-1,2-quinone	57	100	83	(34)
Anthracene-1,4-quinone	100	21	55	(34)
Anthracene-9,10-quinone	100	64	47	(34)
Phenanthrene-9,10-quinone	35	100	52	(34)
Benz(a)anthracene-7,12-quinone	100	43	46	(34)
Benzo(b)naphtho(2,3-d)thiophene-6,11-quinone	100	40	34	(34)
Chrysene-5,6-quinone	19	100	28	(34)
Chrysene-1,4-quinone	100	20	28	(34)
Aceanthrylene-1,2-quinone	58	100	83	(34)
Benzo(a)pyrene-1,6-quinone	100	34	16	(34)
Benzo(a)pyrene-3,6-quinone	100	24	17	(34)
Benzo(a)pyrene-4,5-quinone	72	100	25	(34)
Benzo(a)pyrene-6,12-quinone	100	71	20	(34)
Benzo(a)pyrene-7,10-quinone	100	10	11	(25)*
Benzo(a)pyrene-11,12-quinone	48	100	—	(25)*
Benzo(e)pyrene-4,5-quinone	47	100	40	(34)

*recorded

at

25

eV

an elimination of CHO, sulfur, CHS, SO, H₂SO₂, and CHSO₂, respectively from sulfones, and (M-16) (elimination of oxygen) from sulfoxides. The latter fragment is also found in the mass spectra of N-oxides and epoxides. Tables 16 and 17 present MS data on some thiaarenes.

(c) Applications

GC/MS has been successfully applied to biochemical studies of PAH-metabolism. Stoming *et al.* /24,40/ have devised a gas chromatographic assay of epoxide hydrolase activity with 3-methylcholanthrene-

TABLE 16

Main mass spectrometric fragments and their relative intensities of some thiarene sulfonates (recorded at 80 eV)

Metabolite	M	Relative intensities (%)						References
		M-29	M-32	M-45	M-43	M-66	M-77	
Benzo(b,naphtho(1,2-d)thiophene-7,7-dioxide	100	45	34	28	45	18	29	(37)
Benzo(b,naphtho(2,1-d)thiophene-11,11-dioxide	100	40	30	28	58	15	47	(37)
Benzo(b,naphtho(2,3-d)thiophene-5,5-dioxide	100	50	60	35	65	40	20	(37)
Triphenylene(4,5,b,d)triopiene-4,4-dioxide	74	13	—	—	100	12	29	(39)*

*recorded at 70 eV

TABLE 17

Main mass spectrometric fragments and their relative intensities of some thiaarene sulfoxides (recorded at 80 eV)

Metabolite	Relative intensities (%)		
	M	M-16	References
Benzo(b)naphtho(1,2-d)thiophene-7-oxide	28	100	(34)
Benzo(b)naphtho(2,1-d)thiophene-11-oxide	34	100	(34)
Benzo(b)naphtho(2,3-d)thiophene-5-oxide	22	100	(34)

11,12-oxide as substrate by determination of the resulting 11,12-dihydrodiol after trimethylsilylation. Bettencourt *et al.* /26/ improved the sensitivity of the method by derivatizing the metabolites with bis-(chloromethyl)tetramethyldisilazane / chloromethyltrimethylchlor-silane / pyridine (2:1:4) and using a highly sensitive electron-capture detector or a CGC/MS combination. Under their experimental conditions concentrations of 1 ng/mL (= 1 pg/ μ L) of the incubation mixture could be detected and evaluated.

Takahashi *et al.* have identified 3-methylcholanthrene related /41/ as well as a broad variety of benzo(a)pyrene metabolites /25/.

In a series of papers Jacob *et al.* have studied the metabolism of a number of PAH with rat liver microsomes. They also studied the inducibility of P450/P448 isocytochromes in rats after pretreatment with various xenobiotics such as PAH, pesticides, and pharmaceuticals. After phenanthrene application they found 9,10-dihydroxy-9,10-dihydrophenanthrene to be the exclusive metabolite of phenanthrene in liver microsomes of untreated rats, whereas pretreatment with PAH and related compounds resulted in different rates of additional oxidation at the 1,2- and 3,4-position. Moreover, secondary metabolism to dihydrodiol epoxides, detected as triols, was observed /28/. Evidence for the formation of the proximate and ultimate carcinogen of phenanthrene was also presented in that paper. A typical phenanthrene metabolite profile after P448 induction with chrysene is given in Fig. 6.

In a study of the rat liver microsomal metabolism of pyrene 1-hydroxypyrene and 4,5-dihydroxy-4,5-dihidropyrene have been ob-

Fig. 6: Profile of phenanthrene metabolites in liver microsomes of chrysene-treated rats recorded after silylation (1 = phenanthrene; 2 = 4-hydroxyphenanthrene; 3 = (trans-9,10-dihydroxy-9,10-dihydrophenanthrene; 4 = trans-1,4-dihydroxy-3,4-dihydrophenanthrene; 5 = 1-hydroxyphenanthrene; 6 = 3-hydroxyphenanthrene; 7 = 2-hydroxyphenanthrene; 8 = K-region triol; 9 = unknown dindriol; 10 = trans-1,2-dihydroxy-1,2-dihydrophenanthrene; 11 = K-region triol; 12 = non K-region triol; 13 = unidentified; 14 = benzofluoranthene (internal standard from 28)).

served to be the main metabolites apart from smaller amounts of two diphenols (tentatively identified as 1,6- and 1,8-dihydroxypyrene) and a triol /31,42/ (see Figure 7).

Several CGC/MS investigations of the metabolism of benz(a)anthracene /29,43,47/ have demonstrated that the molecule can be attacked at all sites by liver microsomes from variously pretreated rats (at the 1,2-, 3,4-, 5,6-, 8,9- and the 10,11-position), whereas microsomes of untreated rats convert benz(a)anthracene almost exclusively to the 10,11-dihydrodiol. The time course of the rat liver microsomal metabolism of benz(a)anthracene /44/ as well as the dose-dependent induction of this metabolism by benzo(k)fluoranthene have been studied by means of CGC/MS /45/.

Two papers report on the rat liver microsomal activation of chrysene using GC/MS for detection and identification of the metabolites /30,46/. Oxidation preferentially takes place at the 1,2- and 3,4-position. The formation of secondary metabolites including the ultimate carcinogen of chrysene has been reported /30/. Figure 8 presents a typical gas chromatogram of a chrysene metabolite profile in liver microsomes of PCB-treated rats.

GC/MS analysis of the benzo(e)pyrene metabolite profile as formed by rat liver microsomes showed the formation of 1- and 3-hydroxybenzo(e)pyrene and 4,5-dihydroxy-4,5-dihydrobenzo(e)pyrene as well as the proximate carcinogen of this PAH. The ultimate carcinogen (9,10-dihydroxy-11,12-epoxy-9,10,11,12-tetrahydrobenzo(e)pyrene), however, is not found which may explain the inefficiency of this PAH as a carcinogen. Pretreatment of the animals by various xenobiotics resulted in more complex patterns and induced also the formation of the 9,10-dihydrodiol /32,33/. A liver microsomal benzo(e)pyrene metabolite profile from benzonaphthoflavone-treated rats is presented in Figure 9.

Recently, the rat liver microsomal metabolism of indeno(1,2,3-cd)pyrene has been investigated by CGC/MS /34/ which confirmed the findings obtained with HPLC /18,19/.

The metabolism of some heterocyclic polycyclic aromatic compounds such as azaarenes and thiaarenes has also been studied by CGC/MS. Conversion of benz(a)- and benz(c)acridine to various phenols, dihydrodiols and triols has been reported for both of them, whereas

Fig. 7: Profile of pyrene metabolites in liver microsomes of untreated rats recorded after silylation (1 = pyrene; 2 = *trans*-4,5-dihydroxy-4,5-dihdropyrene; 3 = 1-hydroxypyrene; 4 = K-region triol (tentatively identified as 1,4,5-trihydroxy-4,5-dihdropyrene); 5 = dihydroxy-pyrene (tentatively identified as 1,8-dihydroxy-pyrene); 6 = dihydroxy-pyrene (tentatively identified as 1,6-dihydroxy-pyrene) (from 31).

Fig. 8: Profile of chrysene metabolites in liver microsomes of PCB treated rats recorded after silylation (1 = *trans*-5,6-dihydroxy-5,6-dihydrochrysene; 2 = chrysene; 3 = *trans*-3,4-dihydroxy-3,4-dihydrochrysene; 4 = 3-hydroxychrysene; 5 = 1-hydroxychrysene; 6 = *trans*-1,2-dihydroxy-1,2-dihydrochrysene; 7 = 2,3,4-trihydroxy-3,4-dihydrochrysene; 8 = 1,2,3-trihydroxy-1,2-dihydrochrysene; 9 = benzo(ghi)perylene (internal standard) (from 30).

Fig. 9: Profile of benzo(e)pyrene metabolites in liver microsomes of benzonaphthoflavone-treated rats recorded after stimulation (1 = chrysene (internal standard); 2 = benzo(e)pyrene; 3 = *trans*-4,5-dihydroxy-4,5-dihydrobenzo(e)pyrene; 4 = *trans*-9,10-dihydrobenzo(e)pyrene; 5 = 3-hydroxybenzo(e)pyrene; 6 = not identified; 7 = 1-hydroxybenzo(e)pyrene; 8 = *trans*-1,2-dihydrobenzo(e)pyrene (from 33)).

N-oxidation only was observed in the case of benz(c)acridine /35,36/. A similar CGC/MS study with 4 different thiaarenes (benzo(b)naphtho-(1,2-d)-, -(2,1-d)-, and -(2,3-d)thiophene as well as triphenyleno(4,5-bcd)thiophene) demonstrated that the compounds are preferentially converted to sulfoxides and sulfones by rat liver microsomes /37/.

GC/MS has also been used in studying PAH-metabolism in cell and tissue cultures /48,49/. Apart from the formation of phenols and dihydrodiols, a methyl-transfer was detected by GC/MS in the case of the substrate benz(a)anthracene with lung cell cultures (see Figure 10). This extends the findings with rat liver microsomes and shows that, as expected, additional enzyme systems for the primary metabolism of PAH exist in complete cell systems.

Fig. 10: Profile of benz(a)anthracene metabolites in fetal Syrian hamster cells after silylation (1 = benz(a)anthracene; 2 = 12-methylbenz(a)anthracene; 3 and 4 = not identified; 5 = 11-hydroxybenz(a)anthracene; 6 = not identified (M=540); 7 = *trans*-10,11-dihydroxy-10,11-dihydrobenz(a)anthracene; 8 = not identified; 9 = *trans*-8,9-dihydroxy-8,9-dihydrobenz(a)anthracene; 10 = indeno(1,2,3-cd)fluoranthene (internal standard); 11 = 8,9,10-trihydroxy-8,9-dihydrobenz(a)anthracene) (from 49).

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