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OFF-LINE COMBINATION OF LIQUID CHROMATOGRAPHY AND FIELD DESORPTION MASS SPECTROMETRY: PRINCIPLES AND ENVIRONMENTAL, MEDICAL AND PHARMACEUTICAL APPLICATIONS

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CONTENTS

1. INTRODUCTION

The principles of high-performance liquid chromatography (HPLC) and field desorption mass spectrometry (FD-MS) make these methods suitable for the analysis of a wide variety of organic, organometallic and inorganic compounds. In particular, these methods are used for investigations of polar substances of low vapour pressure, which cannot be determined adequately by conventional methods such as gas chromatography (GC) and electron-impact (EI) mass spectrometry. The rapid separation of the individual components of a mixture by HPLC and the high sensitivity and specificity of their detection by FD-MS show that a combination of these methods should be ideally suited to tackling problems in environmental, medical and pharmaceutical research.

In our opinion, the FD method is difficult and there is no practical and efficient way of transferring the HPLC eluents on-line onto the emitter without increasing the technical complexity considerably. Hence these methods should be used in the off-line mode, as was first reported in 1973[1].

Fig. 1 shows schematically the off-line combination of HPLC and FD-MS. Chromatograms are obtained by recording the separated components of the mixture with a suitable detector. Alternatively, the HPLC fractions can be sampled and trans-

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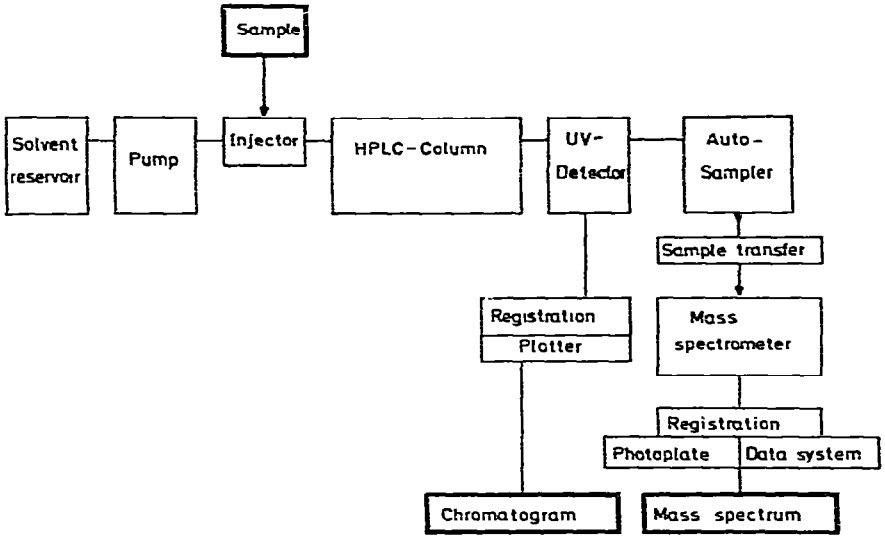

Fig. 1. Schematic diagram of a high-performance liquid chromatograph in off-line combination with a mass spectrometer. After chromatographic separation, the compounds can be recorded by conventional UV, refractive index or electrochemical detection (chromatogram) and/or stored in the autosampler. Identification, purity control and quantification are performed by the mass spectrometer using photographic or electrical (data system or multi-channel analyser) recording

ferred to the mass spectrometer for identification or confirmation of the separated substances or for purity control of products. The off-line combination of HPLC and FD-MS can be used to investigate steroids[1], vitamins[2], alkaloids[3], dyes[4], natural porphyrins and chlorophyll derivatives[5], herbicides[6,7], ginsenosides[8], oligonucleotides[9,10], drugs[11–14] and endogenous compounds[15].

Fig. 2 shows schematically some of the possible applications of combined HPLC and FD-MS. When the HPLC detector is not sensitive enough, FD-MS can be used directly as a detector, as for example in the investigation of the anti-cancer drug cyclophosphamide and its metabolites[11,12]. If similar substances are present in a mixture in high concentrations, an appropriate solvent extraction is sufficient for identification under FD conditions. Compounds in low concentrations or in very complex matrices can be rapidly purified by HPLC[13]. The combination of HPLC and FD-MS employing internal standards is also used for quantitative determinations[11,12,15]. Because of their identical chemical behaviour, compounds and their stable isotope-labelled analogues appear at the same retention times in the chromatogram and can be sampled in the same fraction of eluent. This review illustrates some characteristic analytical applications of the off-line combination of HPLC and FD-MS in medical, environmental and pharmaceutical investigations.

2. MEDICAL APPLICATIONS

2.1. Identification of barbiturates from extracts of urine, stomach fluid and tissues of liver and kidney

An example of the purification and identification of compounds in complex

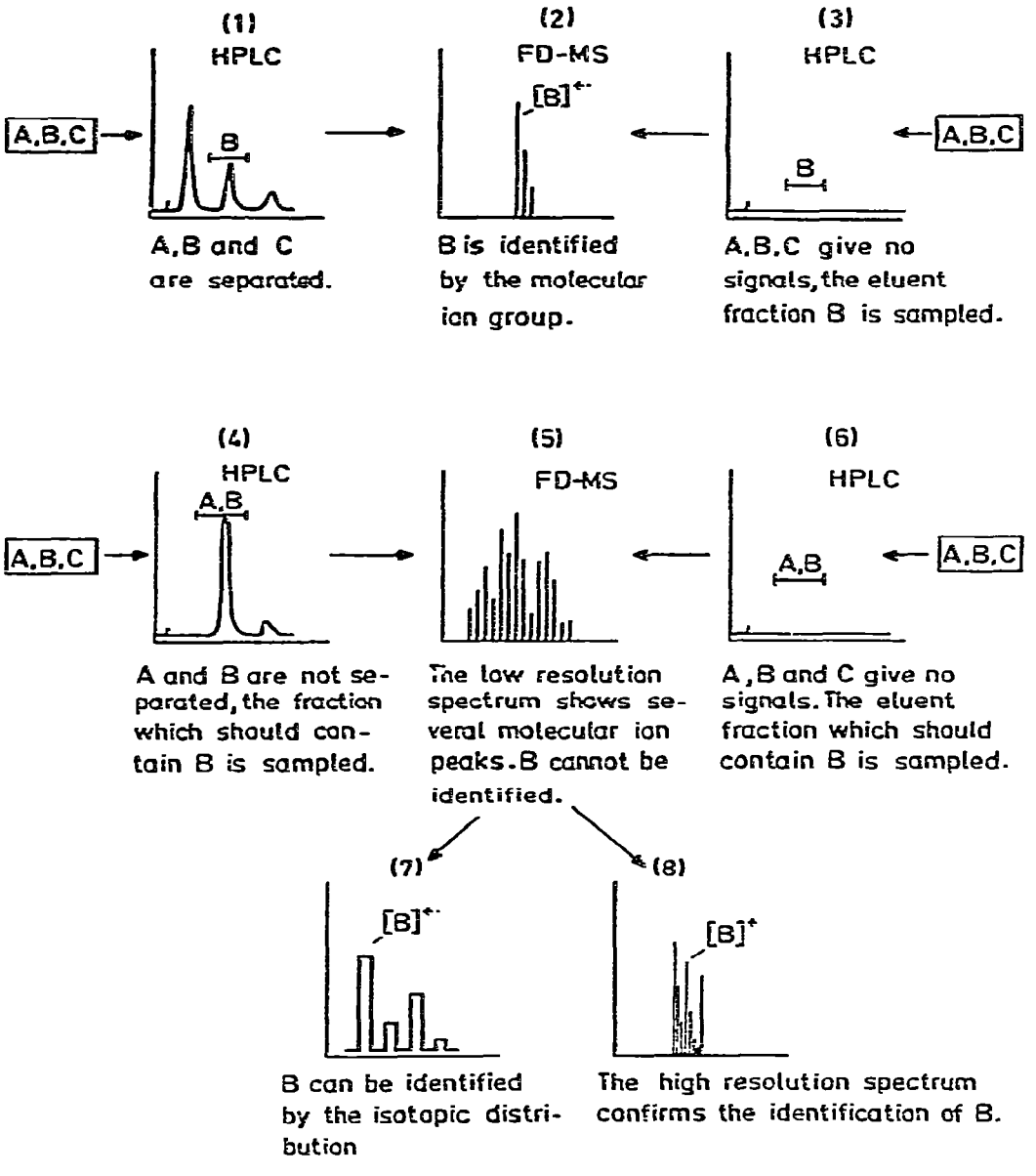

Fig. 2.

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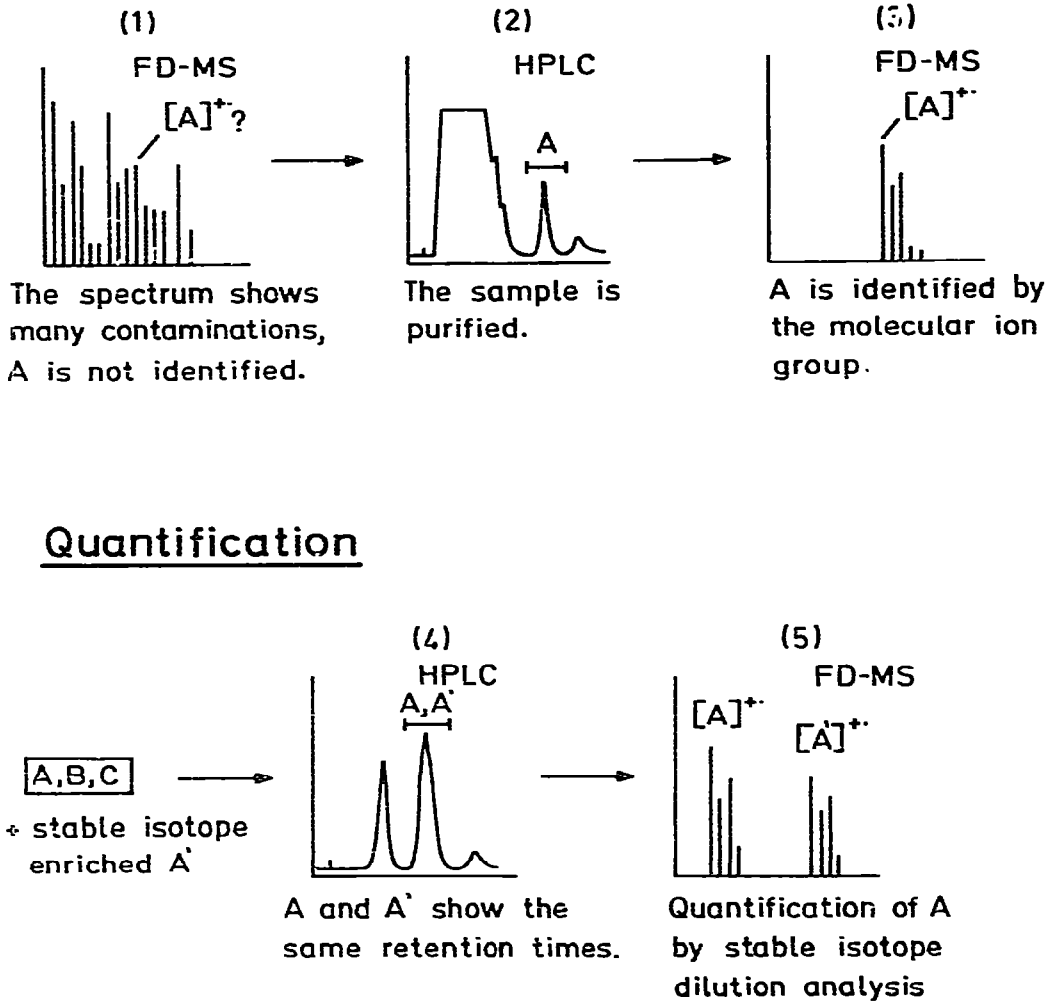

Fig. 2. Schematic diagram of the feasible application modes for the combination of HPLC and FD-MS. (a) Chromatographically separated compounds (1) can be identified or confirmed by FD-MS (2). If the HPLC detector shows no signals (3), FD-MS serves directly as a detector. If a chromatographic peak consists of several components (4) or the chromatogram shows no peaks (6), the electrically recorded FD mass spectrum (5) serves for detection. The substance of interest can be identified either by the isotopic distribution (7) or the high-resolution spectrum (8). (b) If a compound cannot be identified unambiguously from the mass spectrum because the sample is too highly contaminated (1), it can be purified by HPLC (2) and then identified by FD-MS (3). The quantification of a compound A by stable isotope dilution analysis (5) is possible, because A and its stable isotope-enriched analogue A* show the same retention time (4) and can be sampled in the same fraction of eluent.

samples by HPLC has been given in an investigation of barbiturates in human body fluids and tissues[13]. In order to evaluate the feasibility of using combined HPLC and FD-MS for the analysis of barbiturates in physiological fluids, test mixtures were first investigated. Samples of 25 ml of urine containing phenobarbital, hexobarbital and heptabarbital (100 ml of urine with 1 mg of each barbiturate) were extracted with methylene chloride, concentrated under vacuum and a mixture of *n*-hexane and acetonitrile was added to the residue. The separated acetonitrile phase was used for HPLC. In order to identify the barbiturates in the individual fractions, ten 50-μl aliquots of the urine extract were injected into the chromatograph (Siemens S100) and fractionated. The appropriate fractions were then analysed with an FD mass spectrometer (Varian-MAT 731). Barbiturates were identified by comparing their chromatographic retention times with those of corresponding standards and by mass spectral detection of their molecular ions in the collected HPLC fractions.

This procedure was used to investigate two body fluid extracts, a gastric fluid extract from a case of lethal barbiturate poisoning and a urine extract from a case of barbiturate intoxication. The samples were purified by HPLC. Using UV detection, the chromatogram of the gastric fluid extract showed three peaks, which were collected by the fraction collector and investigated by FD-MS. By comparing the retention times, these peaks could be ascribed to phenobarbital, amobarbital and secobarbital. The FD-MS spectra of the HPLC fractions confirmed these findings. Barbital was identified in the urine in the same manner.

When, for instance in forensic investigations, sufficient urine or stomach contents were not available, or when the analysis of these body fluids gave negative results, tissue extracts were investigated. Barbiturates were identified in specimens from two autopsy cases by HPLC and FD-MS as follows. The samples were concentrated acidic chloroform extracts of human liver and kidney, prepared in the Institute of Forensic Medicine of the University of Bonn by the Stas–Otto procedure[13]. In both cases urine was not available and the examination of stomach contents did not reveal the presence of toxic substances. The crude extracts were pre-purified and

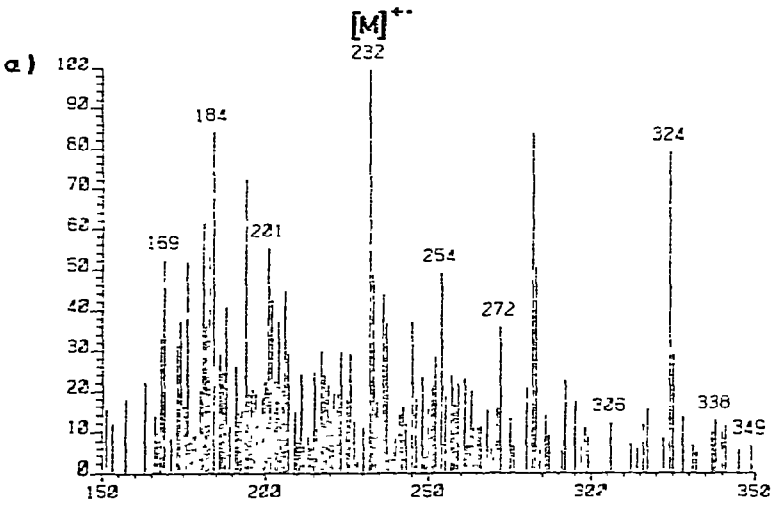

Fig. 3.

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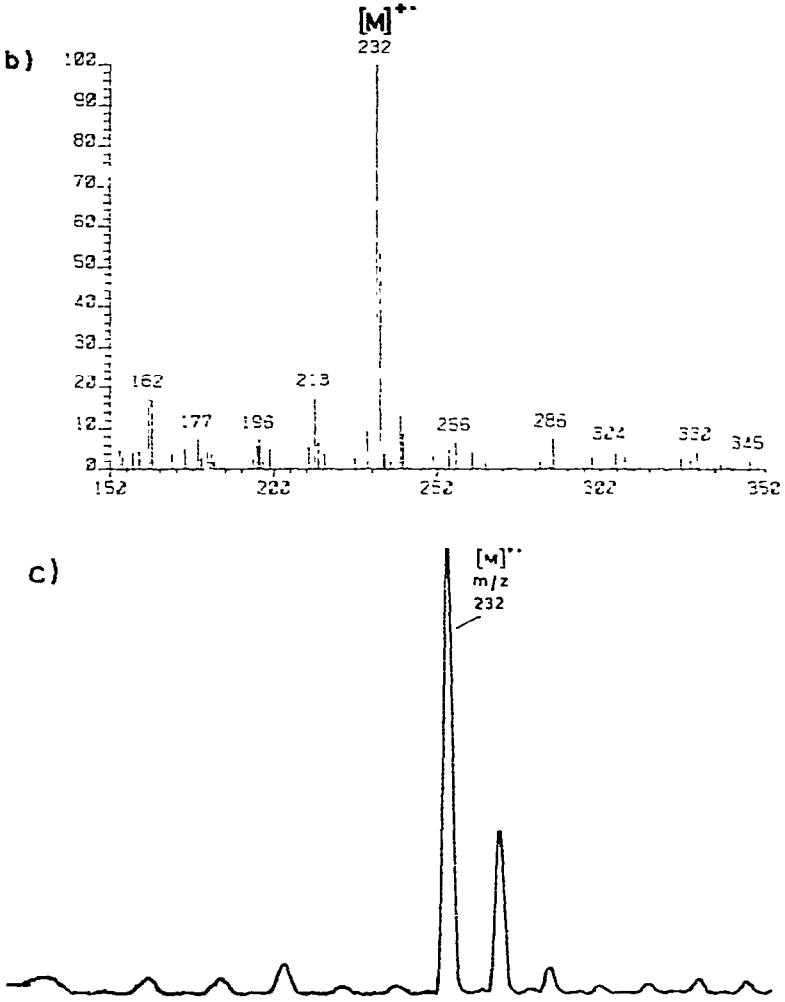

Fig. 3. FD mass spectra of phenobarbital[13] (a) After pre-purification, 25 μl of the crude extract of kidney tissue were applied to the FD emitter and the mass spectrum was recorded electrically using the data system (Varian SS 200). (b) Collection of the fraction "under" the HPLC peak and FD-MS. (c) Part of the collected HPLC fraction was used for recording of the isotopic distribution with the multichannel analyser; 13 scans were accumulated in the range 2^{10}.

dissolved in 25 ml of acetonitrile before HPLC. The chromatograms of both samples showed an intense peak with a retention time of 7.1 min; under the conditions defined for HPLC this retention time corresponds to the retention time of phenobarbital. By comparing the HPLC peak heights of the samples with those of phenobarbital standards, the amount of the barbiturate for one injection was estimated to be 280 ng in the liver extract and 600 ng in the kidney extract.

The effluents under these HPLC peaks were sampled by the fraction collector and investigated separately by FD-MS in order to confirm the identity of the drug. As shown in Fig. 3b for the effluent of the liver extract, the electrically recorded FD

spectrum in the mass range m/z 150–350 exhibits the molecular ion of phenobarbital at m/z 232 with high relative abundance (base peak). With two exceptions, namely m/z 162 and 213, all other signals are below 10% relative abundance. In contrast, the FD spectrum of 25 μl of the pre-purified extract obtained without performing HPLC first (Fig. 3a) shows a multitude of abundant ion signals. Owing to the soft ionization mode and judging from the numerous examples of mixture analysis by FD-MS, it can be assumed that most of these ions represent molecular ions of impurities. Thus the effectiveness of the clean-up by HPLC is demonstrated convincingly. In addition, there was still enough sample material available to record a smaller mass range with the multi-channel analyser so as to obtain a clear picture of the molecular ion group (Fig. 3c). The isotopic pattern of this group provided further evidence for the correct assignment of the barbiturate. The final confirmation was, of course, given by the high-resolution data from which accurate masses of the molecular ions of the barbiturates could be established.

2.2. Determination of cyclophosphamide and some of its metabolites in body fluids

For the identification and quantitative determination of cyclophosphamide (CP) and its metabolites 4-ketocyclophosphamide (4-keto-CP) and carboxyphosphamide (carboxy-P) in body fluids of multiple sclerosis patients, the combination of HPLC and FD-MS has been used[11,12].

Cyclophosphamide (Endoxan) is widely used in the treatment of cancer[16] and rheumatoid arthritis[17] and is also used as an immunosuppressant in organ transplantations[18]. Numerous investigations have been performed to characterize the biologically active metabolites and elucidate the mechanism of action of this important drug[19–26]. For some years, CP has also been used effectively in the treatment of multiple sclerosis[27,28].

In preliminary investigations, the detection limit of CP in a standard solution determined by FD-MS was $5 \cdot 10^{-11}$ g with a signal-to-noise ratio of 20:1 and a sensitivity of $1 \cdot 10^{-11}$–$2 \cdot 10^{-11}$ C/μg.

The identification and quantitative determination of CP and its metabolites (the latter are present at even smaller concentrations in body fluids) in a very complex matrix are often impossible without prior purification of the sample. Fig. 4a shows the FD mass spectrum of a chloroform extract of a serum sample. While the peak at m/z 260, due to the molecular ion peak of CP, shows the highest intensity in the FD spectrum of the pure compound[11], CP cannot be identified in the spectrum of the crude extract. For purification of the sample HPLC is used, mainly because the substances are too polar for analysis by GC–MS without derivatization. CP, 4-keto-CP and carboxy-P cannot be recorded using conventional UV detectors for HPLC. The detection limit for CP employing a variable-wavelength UV detector at 210 nm is 5 μg, for 4-keto-CP 200 ng and for carboxy-P 700 ng (referring to amounts of substance injected on to the column). Because its sensitivity is better by a factor of $4 \cdot 10^3$–10^5, FD-MS is used directly as a detector for HPLC. In other words, although the chromatogram shows no signal, the compound under investigation is sampled "blindly" according to the retention time found for a more concentrated solution of the standard. Following extraction, the sample is separated on the HPLC column filled with RP-C18 using acetonitrile–water as the mobile phase. The separated fraction, containing the compound under investigation, is measured by FD-MS (Fig. 4b).

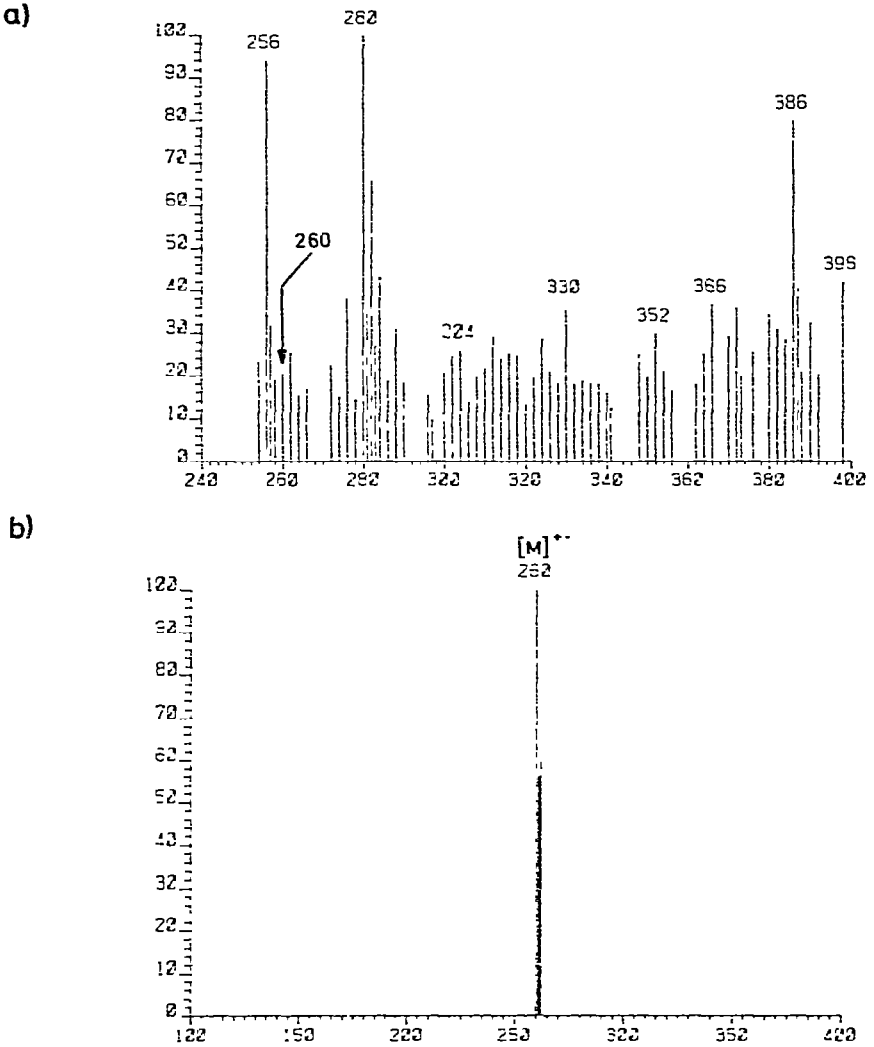

Fig. 4. (a) Electrically recorded FD mass spectrum of a chloroform extract of serum. Cyclophosphamide cannot be identified unambiguously. (b) FD mass spectrum after purification by HPLC[11]. The spectra were obtained with a Varian-MAT 731 mass spectrometer at a heating current between 0 and 10 mA. The plots were obtained from a Statos 33 printer/plotter using the Varian-MAT SS 200 data system.

Quantitative determinations were performed using isotope dilution analysis. Deuterated analogues, $[^2H_{10}]$CP, $[^2H_8]$4-keto-CP and $[^2H_8]$carboxy-P, were used as they appear at the same retention times after HPLC and can be collected in the same fractions as their unlabelled analogues. In this way the time-dependent excretion of unchanged CP in the urine of six patients over a period of 24 h was determined. The patients had received 400 mg of CP per day in four doses of 100 mg each. Within 24 h, between 5 and 10% of the daily dose of unchanged CP were excreted through the kidney. The course of the CP excretion shows several maxima, each occurring 2–3 h after administration of a dose of 100 mg of CP and decreasing very quickly. The

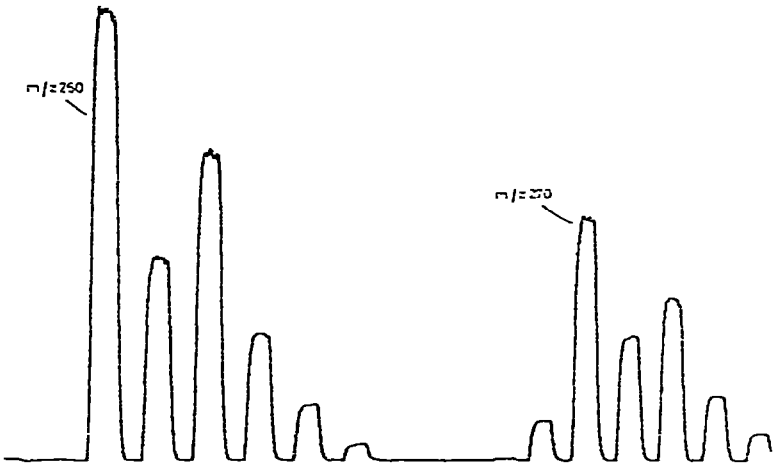

Fig. 5. Isotopic pattern of cylophosphamide and cyclophosphamide-d_{10}, isolated from cerebrospinal fluid, obtained by FD-MS and accumulation of 27 scans with a multi-channel analyser.

highest amounts of CP were excreted at noon and the lowest at night, in accordance with the different activities of the human organs by day and night. Further, there is a correlation between the production of urine and the amounts of CP excreted. The total amounts of CP excreted increase with enhanced urine production.

Fig. 5 shows the FD mass spectrum of the isotope dilution analysis of CP and [$^2H_{10}$]CP isolated from cerebrospinal fluid. This assay revealed that the concentrations in serum and cerebrospinal fluid were of the same order of magnitude, between 270 and 2500 ng/ml. The results indicate that CP passes the blood–spinal barrier freely. This implies that if quantitation of CP in serum is performed, then the concentration of the drug in the cerebrospinal fluid can be derived and the patient need not undergo cerebrospinal fluid sampling.

It is possible that CP migrates into the brain tissue and that the levels of spinal fluid also reflect the CP levels in the brain. This may explain the special effect of CP on the pathological IgG-synthetizing system in the brain of multiple sclerosis patients[28]. The results of quantitative determinations of CP, 4-keto-CP and carboxy-P from urine of one patient were as follows: CP, 39.5 μg/ml; 4-keto-CP, 0.79 μg/ml; and carboxy-P, 23.2 μg/ml. The ratio of CP to carboxy-P is in good agreement with results obtained from previous investigations of tumour patients[29,30], according to which the concentration of carboxy-P is about 30–50% of the concentration of CP. In the same work, a concentration of 4-keto-CP of 10–20% of the CP concentration was reported, whereas the level of 4-keto-CP determined in our study is much lower.

The duration of the complete assay, including sample preparation and quantitative FD measurements, was 20–30 min. Repeated analyses showed that the precision of the determination at the parts per 10^9 level of the parent drug was about ±5%

2.3. In vivo determination of free phenylalanine and tyrosine in plasma

Combined HPLC and FD-MS has been used successfully for the highly specific detection and reliable quantification of free amino acids in human plasma[15]. In par-

ticular, for a variety of polar and labile amino acids this combination has advantages over GC–MS, as evaporation of the sample is not required.

For the isolation of free L-tyrosine and L-phenylalanine from plasma samples, cation-exchange chromatography was used, followed by HPLC on RP-18. The separated components were detected with a UV detector at 336 nm, collected in an autosampler and transferred to the mass spectrometer (VG Micromass ZAB-1F) for identification. The FD sensitivity was increased to about 10^{-10} C/μg and the reproducibility of the results was improved by using the Dns derivatives of L-tyrosine and L-phenylalanine instead of the free acids.

For quantitation, L-[$^2H_7$]phenylalanine and L-[$^2H_7$]tyrosine were added to the plasma sample before purification. The results were obtained by stable isotope dilution and FD-MS. About 100–500 repetitive magnetic scans were accumulated in order to achieve a precision of about ±0.5% in the relative ion abundances. Using this procedure the kinetics of hydroxylation of L-phenylalanine to L-tyrosine was investigated *in vivo*. This is of interest as genetic disorders of the phenylalanine-hydroxylating system are one of the most commonly occurring inborn errors in man[31].

Two healthy volunteers were given oral doses of 25 mg/kg of L-[$^2H_5$]phenylalanine and the hydroxylation kinetics of labelled and unlabelled L-phenylalanine and L-tyrosine were followed over the next 5 h (Fig. 6). Both deuterated L-phenylalanine and deuterated L-tyrosine show a rapid increase up to maximum concentrations 30 min after the oral dose. At this point, the concentration of L-[$^2H_4$]tyrosine was found to be about 15% of the peak concentration of L-

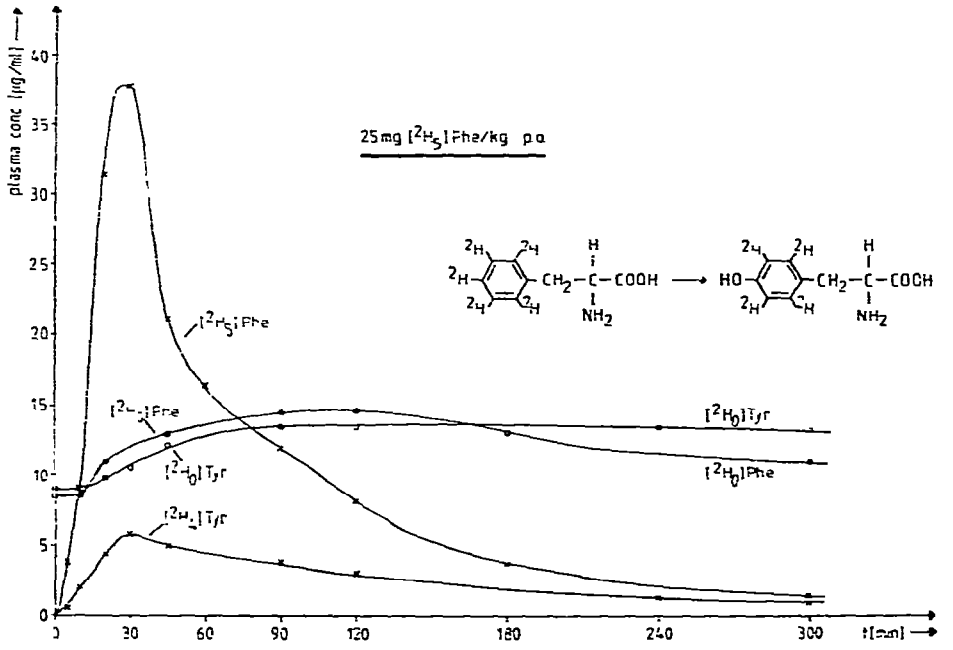

Fig. 6. Time course of the plasma concentrations of L-[$^2H_0$]phenylalanine, L-[$^2H_5$]phenylalanine, L-[$^2H_0$]tyrosine and L-[$^2H_4$]tyrosine in a loading test with 25 mg/kg of L-[$^2H_5$]phenylalanine in a healthy volunteer[15].

[$^2H_5$]phenylalanine. Fig. 6 also indicates that the level of deuterated L-phenylalanine decreases more rapidly than does the corresponding level of deuterated L-tyrosine. Analysis of the elimination of L-[$^2H_5$]phenylalanine according to simple first-order kinetics gave a plasma $t_{1/2}$ of about 1 h compared with the plasma $t_{1/2}$ of *ca.* 1.5–2 h reported for L-[$^2H_0$]phenylalanine[32,33]. This difference is due to the relatively fast exchange between free and protein-bound phenylalanine by which L-[$^2H_5$]phenylalanine is removed from the plasma and replaced by L-[$^2H_0$]phenylalanine[34]. This effect reduces the $t_{1/2}$ value for the labelled molecule compared with the unlabelled species.

The concentrations of unlabelled L-phenylalanine and L-tyrosine, which are also given in Fig. 6, shows a significant increase during the first hour after the intake of the deuterium-labelled L-phenylalanine. The increase in the L-[$^2H_0$]phenylalanine concentration can be explained by the same model discussed above for the elimination rate of L-[$^2H_5$]phenylalanine: Most of the L-phenylalanine that is converted from the free form to a protein-bound form during the first hour of the kinetic measurements is deuterated L-phenylalanine, whereas most of the L-phenylalanine that is released is unlabelled L-phenylalanine. Consequently, the plasma concentration of the unlabelled species increases during the presence of high concentrations of free L-[$^2H_5$]phenylalanine.

The roughly parallel increase in the L-[$^2H_0$]tyrosine concentration obviously follows from simultaneous oxidation of both labelled and unlabelled L-phenylalanine by the phenylalanine-hydroxylating system under the conditions of the loading test. The kinetic behaviour of the four amino acids was identical in the test on the second volunteer. The plasma sample taken at 30 min again showed the highest concentrations for L-[$^2H_5$]phenylalanine and L-[$^2H_4$]tyrosine, which were 22.1 and 5.0 μg ml, respectively. The precision of a single determination was ± 2–3%. With respect to sensitivity, the assay is capable of following the L-[$^2H_4$]tyrosine concentration in plasma at levels down to about 100 ng/ml.

2.4. Investigation of biologically active oligopeptides

HPLC with chemically bonded alkyl stationary phases and a tetraalkylammonium phosphate buffer has been used for the separation of synthetic mixtures of hypothalamic oligopeptides containing from three to thirty-one amino acids[35,36] and neuropeptides in biological tissue[37]. The sensitivity of the UV detector allowed the detection of amounts of peptide down to 5 ng. FD-MS has been employed for the structural elucidation of the compounds and for quantification[37,38].

3. ENVIRONMENTAL APPLICATIONS

3.1. Determination and identification of biocides of the phenylurea, carbamate and thiocarbamate type in surface water

Combined chromatographic and MS investigations have been applied to the reliable identification of biocides in surface water samples from the Rhine and some of its tributaries over a period of 1 year[7].

After an elaborate extract preparation and separation procedure, the compounds were determined by calibration against corresponding test substances. For this purpose, well standardized and reproducible chromatographic conditions are

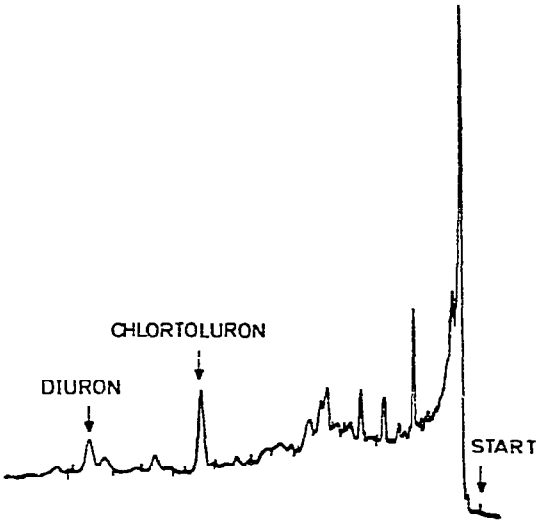

Fig. 7. HPLC trace of sample 2333, D-fraction (20 μl)[6]. Apparatus, Waters M6000A; column, 200 × 4 mm I.D.; stationary phase, Nucleosil RP-C18; mobile phase, methanol–water (47:53); temperature, 30°C; detector, UV at 254 nm.

required and these have been established[6]. The raw extract of a water sample was chromatographed on silica gel to remove most of the interfering substances. Subsequently, the raw extract was separated by gradient elution, starting from *n*-hexane–chloroform (8:2) up to 98% chloroform. Using this technique, a test mixture consisting of ten phenylurea derivatives and two carbamates was split into five fractions. Before HPLC determination, the single fractions were purified further on RP-C8 material. For the HPLC separation and quantitative determination of the phenylurea and carbamate derivatives, two different columns (RP-C18 and RP-C8) are used for separation, in order to optimize the chromatographic parameters of the substances to be determined. Methanol–water mixtures are used as the mobile phase. A UV detector (254 nm) and integrator–calculator system is employed for the detection and determination of the individual substances. Fig. 7 shows the chromatogram of the D-fraction of the test mixture. The recovery of these compounds after the whole procedure, including extraction and preparation of the raw extract, is between 70 and 85%. The precision of the method is ±5% and the accuracy is ±10%.

The reliable identification of trace substances from water samples is not possible from a comparison of the HPLC results with the reference sample chromatograms alone, because the HPLC peak can consist of several compounds [*cf.*, Fig. 2a (5)]. Therefore, confirmation must be obtained from an independent technique. FD-MS offers favourable conditions for the identification of biocides of low volatility from water samples[39]: Soft ionization in a high electric field produces molecular ions of high intensity from highly polar substances. In particular, compounds that cannot be analysed by GC–MS and must be separated by HPLC can be transferred directly and without derivatization to the FD emitter and ionized without a separate evaporation process.

In general, four different methods can be used for recording and evaluating FD

signals in the identification of biocides from pre-purified water-extracts:

(1) Using electrical detection, the FD signals recorded can be observed directly on the oscilloscope of the mass spectrometer. The first intense FD ions of the sample to appear are used for fine adjustment of the mass spectrometer after halting the scan. Model FD investigations of phenylurea and carbamates have shown that the best anode temperature for desorption is between 10 and 20 mA e.h.c.[39]. However, it was also found that the emitter heating current required for the biocide was generally 5–10 mA higher in the water extracts investigated than in the measurements of standard compounds from pure solvents. This is clearly due to the influence of the accompanying inorganic, organic and partly polymeric matrix from the sample. This turns out to be helpful in the following ways. First, no significant part of the biocide to be investigated is lost at the low heating currents (0–5 mA) used for fine adjustment, and secondly, the frequently occurring intense FD signals of the matrix allow optimal adjustment of the FD-MS before desorption of the compound.

In the mass range relevant for the biocides, this method can provide two important preliminary pieces of information. First, if the mass peak of the plant-protective agent assumed on the basis of the HPLC data is not recorded in the heating current interval characteristic for phenylurea and carbamates, the presence of these substances in the water extract can be excluded. Second, if the mass number of the biocide in question is evident, the best anode temperature (BAT) can be determined by observing the dependence of signal intensity on emitter heating current[40]. Hence by recording further FD spectra, one may compensate for the influence of the matrix in the sample and by fractional desorption record only that part of the FD ion current relevant for identification.

(2) In the second step, complete FD spectra in the mass range of interest (from about m/z 50 to 350) are recorded electrically and stored on-line in the data system. The mass spectra so obtained can only be interpreted after complete desorption, as registration is carried out by continuous, repetitive scanning. In most instances, the value of the total ion current during desorption does not give evidence of the presence of the substances of interest, as the ion formation is superimposed by accompanying substances in the complex sample mixture. After calibration of the signals, setting the threshold level, subtraction of background, etc., the FD spectra are recorded individually and displayed on an oscilloscope terminal (Tektronix Type 4010-1 Storage Oscillograph). It is now possible to select the mass numbers corresponding to the intense molecular ions and characteristic fragments and to obtain a first basis for identification of the biocides.

(3) As many biocides of interest contain elements with significant isotopes, such as chlorine and bromine, the isotopic distribution of the molecular ions takes on a particular significance. If a halogen-containing compound was indicated from the HPLC measurement, and this was supported by the distribution of the signals in the molecular ion group in the total spectrum, a narrower mass range (about m/z 200–250 for diuron and chlortoluron in Fig. 8) was selected and recorded with a fast repetitive magnetic scan. The ion signals obtained were accumulated on a multi-channel analyser. Integration over a large number of mass runs then permitted a direct isotope analysis. As has been reported[41], amounts of substances in the microgram range are sufficient for several hundred scans, and a precision of a few tenths of a percent is obtained for the isotope determination. Nevertheless, a limitation of the method is

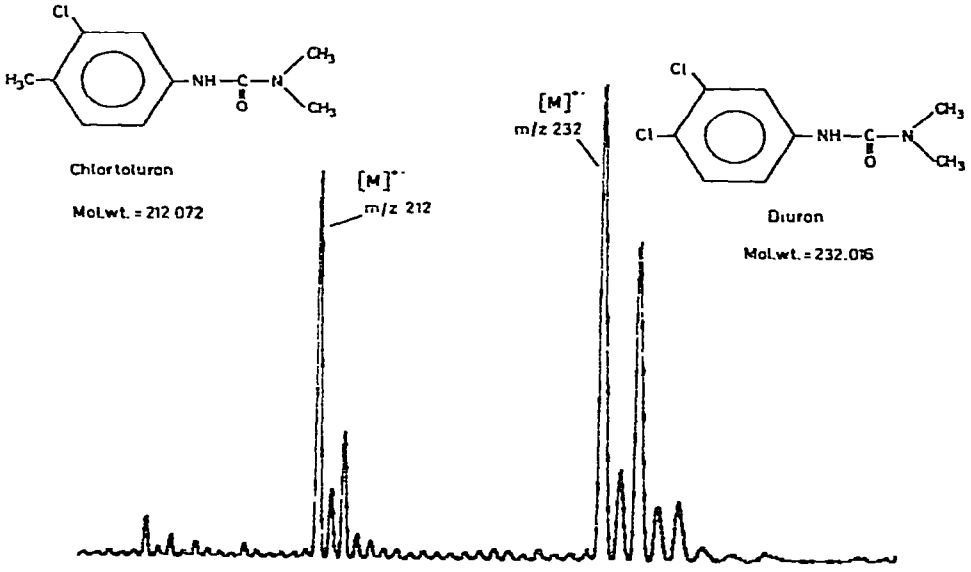

Fig. 8. Isotopic distribution of chlortoluron and diuron from the D-fraction of a water sample from the Rhine after purification by HPLC[14]. Apparatus: double-focusing mass spectrometer (MAT 731). The spectrum was obtained by accumulation on a multi-channel analyser (Varian C-1024) over 74 scans.

that the results for isotope determinations on substances from the extracts cannot be obtained with the same precision as is possible for model compounds[41] or standard biocides from pure solvents[39], because only nanogram amounts are available in the HPLC eluate, the accompanying matrix increases fluctuations in the ion current and interfering field reactions on the surface of the emitter occur. By using integrating recording of FD ions together with selection of a narrow mass range, as described here, not only an indication of the isotopic distribution but also excellent sensitivity are obtained.

(4) The fourth method for identifying biocides is the most specific and provides the most information. While in the previous three stages the FD spectra were recorded at low resolution ($m/\Delta m$ *ca.* 800, at 10% valley definition) and hence the emphasis lay on the detection sensitivity, high-resolution FD-MS[40,42] allows the calculation of the elementary composition of the various ions. Accepting the disadvantage of lower sensitivity, this method is used as the final step, as it usually requires the whole of the remainder of the HPLC extract. This method has been used successfully for the identification of herbicides in Chinese river water[43]. One has the choice of either determining the accurate mass of a prominent FD ion, *e.g.*, the molecular ion, with the electrical detection system and the help of the peak-match technique[44,45] or of recording high-resolution data for the entire mass range (*e.g.*, m/z 20–500) on a photoplate. Although the correct element composition identifies the cations reliably and definitely, the amounts of extracts from slightly polluted water are not always sufficient for photographic detection.

The results of applying combined HPLC–FD-MS to the analysis of herbicides of the phenylurea type in Rhine water are as follows. Chlortoluron, diuron, linuron and metoxuron were found in nearly all of the samples examined over a period of 1

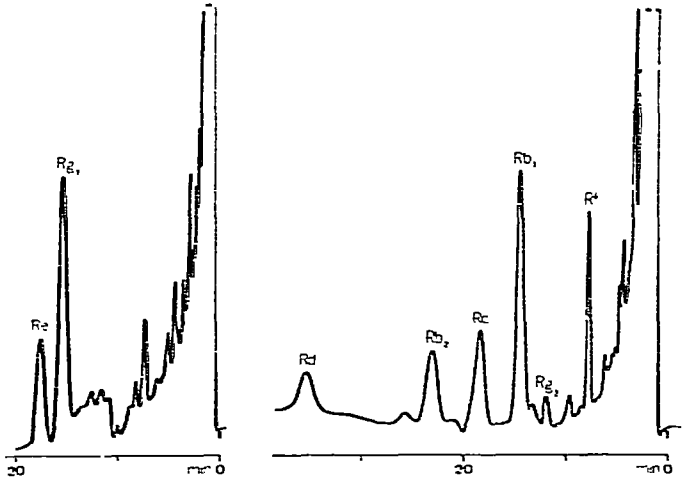

Fig. 9. HPLC trace of ginseng extract (G115S). 7 μl for A, 12 μl for B[8] Eluent A, acetonitrile–water (29·71), 2 ml/min; eluent B, acetonitrile–water (18:82), 4 ml min. Rb_1, 13 μg; Rb_2, 8 μg; Rc, 11 μg; Rd, 4 μg; Re, 17 μg; Rf, 2 μg; Rg_1, 11 μg; Rg_2, 1 μg

year. Metobromuron and monolinuron were identified in only half the samples. Other substances were detectable sporadically or not at all. In the tributaries of the Rhine, only diuron was found in all samples and all other carbamate herbicides were detected only in certain samples[7].

4. PHARMACEUTICAL APPLICATIONS

4.1. Identification of ginsenosides from Panax ginseng

HPLC has been used for the separation and determination of ginsenosides in plant materials and various galenical preparations[8,46,47]. Purity control of the compounds separated by HPLC is important mainly for substances to be determined in complex mixtures, such as body fluids or plant extracts. The possibilities and limits for the identification of steroid saponins from extracts of *Panax ginseng* Meyer (G115 and G115S; Pharmaton, Lugano-Bioggio, Switzerland) in eluted fractions from HPLC (see Fig. 9) by various off-line methods have been described[8]. The methods used included high-performance thin-layer chromatography (HPTLC), multiple internal reflection (MIR) infrared (IR) spectroscopy and FD-MS. In HPTLC, the ginsenosides were separated with a running distance of 6.5 cm, the detection limit being 0.2 μg. In MIR-IR, spectra were obtained with 20 μg of ginsenoside. FD-MS not only permits the identification and molecular weight determination of the underivatized ginsenosides, but also yields important information about the sequence of the sugar moieties in the molecule. An FD mass spectrum was obtained with less than 1 μg of ginsenoside. Fig. 10 shows the FD mass spectrum of the HPLC fraction containing the ginsenoside Rc.

In order to survey the thermally induced fragmentation that can be generated and how these fragments can be explained, this ginsenoside was investigated in that part of the desorption process which gave the most intense ion currents for structur-

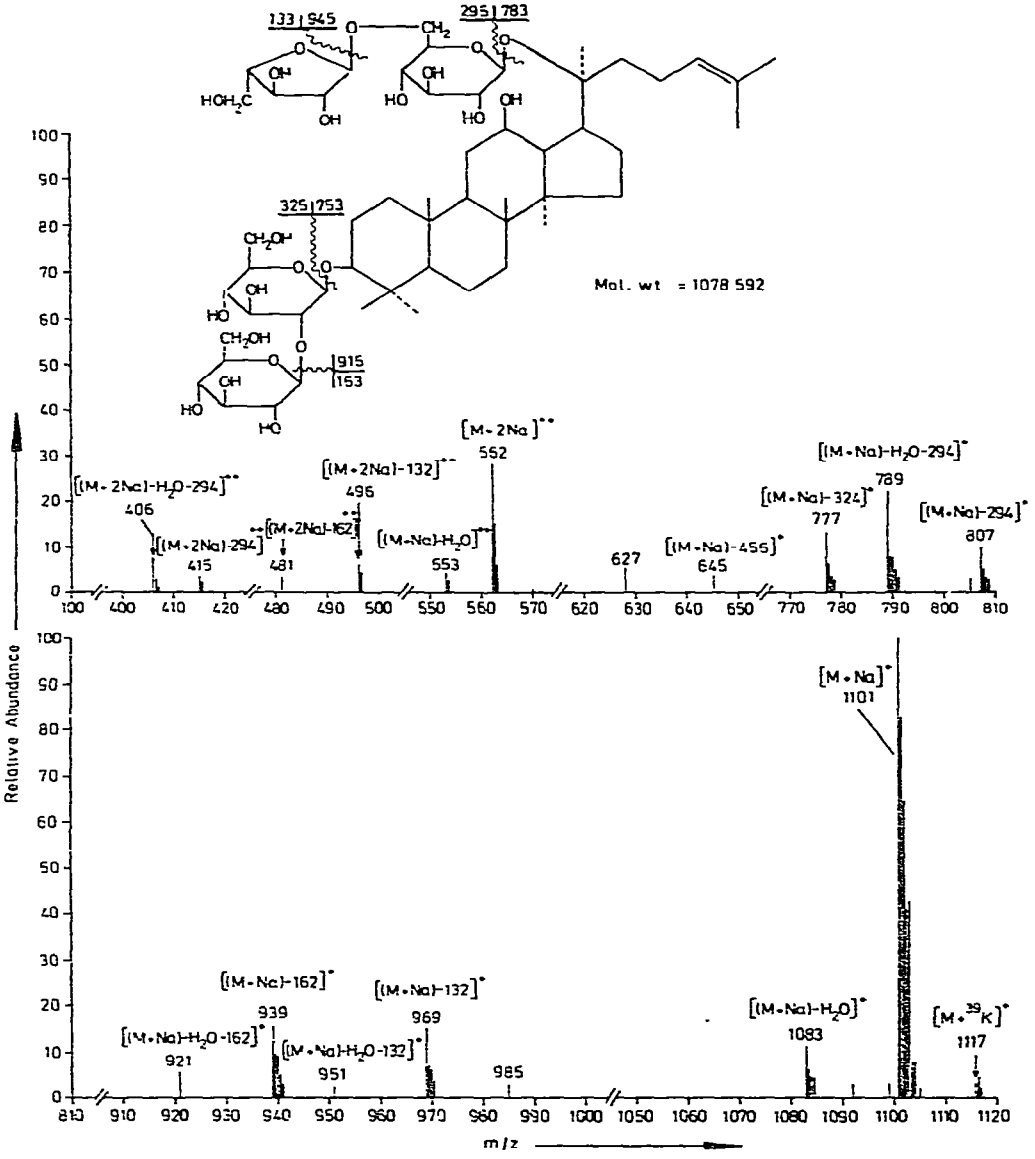

Fig. 10. FD mass spectrum of the HPLC fraction containing compound Rc[8]. Within the emitter heating current interval from 25 to 30 mA eight FD spectra were recorded and averaged. The base peak intensity is 57,776 counts and the background level (noise) is about 50 counts.

ally significant fragments. The molecular weight of this ginsenoside is obtained unambiguously from three series of FD ions, $[M + {}^{39}K]^+$ at m/z 1117, $[M + Na]^+$ at m/z 1101 and $[M + 2Na]^{2+}$ at m/z 562. Loss of one glucose unit (-162) explains the ion at m/z 939 and loss of two glucose units that at m/z 777. Elimination of arabinose (-132) is indicated by the ion at m/z 969 and the loss of one arabinose and one glucose unit by the ion at m/z 807.

TABLE I

DETERMINATION OF THE MOLECULAR WEIGHT AND ASSIGNMENT OF STRUCTURALLY SIGNIFICANT SIGNALS FOR CONFIRMATION OF THE SUGAR SEQUENCE IN THE HPLC FRACTION OF THE GINSENOSIDE Rc BY FD-MS[8]

Type of ion	*Relative abundance**	*Accurate mass*
$[M + K]^+$	±	1117.556
$[M + Na]^+$	∓ − − + −	1101.582
$[(M + Na) - H_2O]^+$	+	1083.573
$[(M + K) - Ara]^+$	±	985.514
$[(M + Na) - Ara]^+$	−	969.540
$[(M + Na) - H_2O - Ara]^+$	±	951.529
$[(M + Na) - Glc]^+$	±	939.529
$[(M + Na) - H_2O - Glc]^+$	±	921.519
$[(M + Na) - Ara - Glc]^+$	−	807.487
$[(M + Na) - H_2O - Ara - Glc]^+$	∓ ∓	789.427
$[(M + Na) - (Glc - Glc)]^+$	±	777.477
$[(M + Na) - H_2O - (Glc - Glc)]^+$	+	759.466
$[(M + Na) - (Glc - Glc) - Ara]^+$ $[(M + Na) - (Ara - Glc) - Glc]^+$	∓	645.434
$[(M + Na) - H_2O - (Ara - Glc) - Glc]^+$ $[(M + Na) - H_2O - (Glc - Glc) - Ara]^+$	±	627.424
$[M + 2Na]^{2+}$	− − ±	562.286
$[(M + 2Na) - H_2O]^{2+}$	− −	553.281
$[(M + 2Na) - Ara]^{2+}$	±	496.265
$[(M + 2Na) - Glc]^{2+}$	±	481.266
$[(M + 2Na) - Glc - Ara]^{2+}$	±	415.238
$[(M + 2Na) - H_2O - Glc - Ara]^{2+}$	∓	406.233

* The relative abundances for electric detection are given for five degrees: 0–20 °$_o$ = −; 20–40 °$_o$ = ± ±; 40–60 °$_o$ = + ∓ ∓; 60–80 °$_o$ = + + + ±; 80–100 °$_o$ = ± + ∓ + +.

Elimination of water from the aglycone gives intense ion signals at m/z 1083 and 789. Several doubly charged ions from the attachment of two sodium cations are detected. The accurate masses of all types of ion found and their interpretations are given in Table I. From the other HPLC fractions similar results were obtained, as shown in Fig. 9[8].

The analysis time required for the FD investigation of an HPLC fraction containing a high-molecular-weight glycoside is about 1 h, including sample preparation, FD measurement, data processing, output, evaluation and interpretation. Of course, in general the interpretation of the FD mass spectra obtained is the most time-consuming step. However, comprehensive experience in analysing the FD signals of glycosidic natural products[48–53], and in particular applying the hypothesis of the analogy of sugar cleavages by acidic solvolysis and FD-MS, has facilitated the evaluation of unknown, physiologically active substances[52]. The predictive value of the method for molecular determinations and sequencing has been demonstrated convincingly[53].

4.2. Identification and purity control of protected deoxyribonucleotides[9,10]

Synthetic oligo- and polynucleotides have a wide field of application, especially

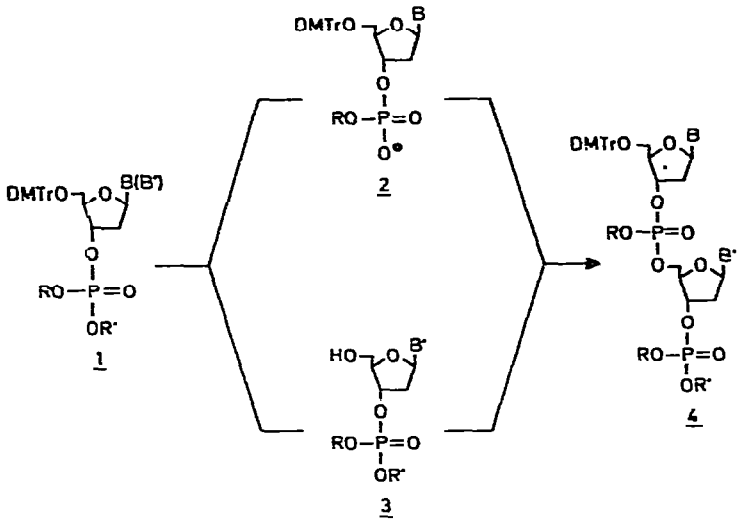

Fig. 11. Synthesis of dinucleotide building blocks for the preparation of polynucleotides[10]. B = B' = protected nucleobases; R = *p*-chlorophenyl-; R' = *β*-cyanoethyl-; DMTr = *p,p'*-dimethoxytrityl-.

in molecular biology, genetic engineering and related areas. Currently, the predominant synthetic method is the "modified triester method", developed by several groups in recent years[5-4]. The key intermediates most commonly used in this approach are the blocked monomers 1, which can easily be converted into the functionalized monomers 2 and 3 by alkali and acid treatment, respectively. Monomers 2 and 3 can be combined to give a standard set of dinucleotide blocks 4 (Fig. 11). These are terminally

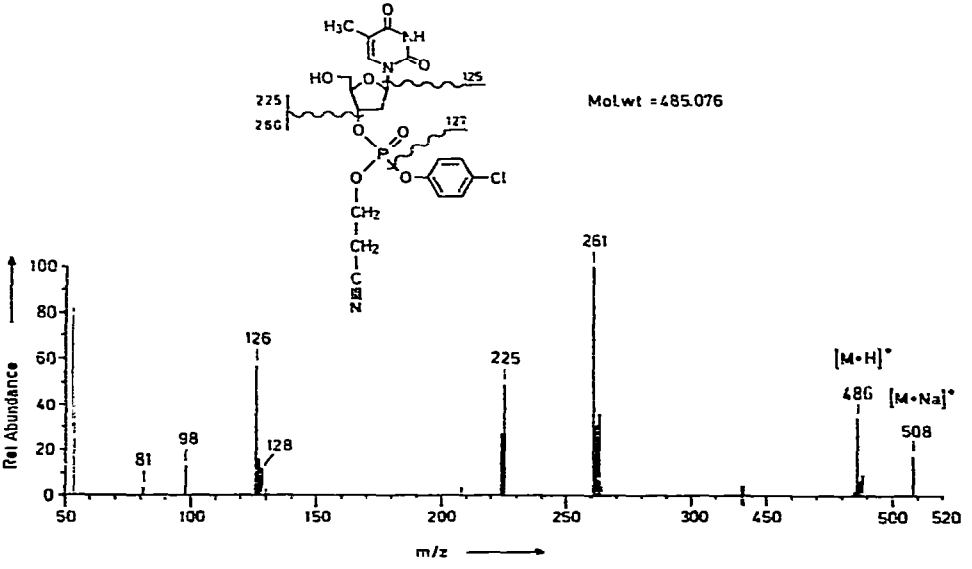

Fig. 12. FD mass spectrum of a partially protected, functionalized mononucleotide after HPLC separation. Average of eight magnetic scans by the data system. The emitter heating current was between 20 and 22 mA.

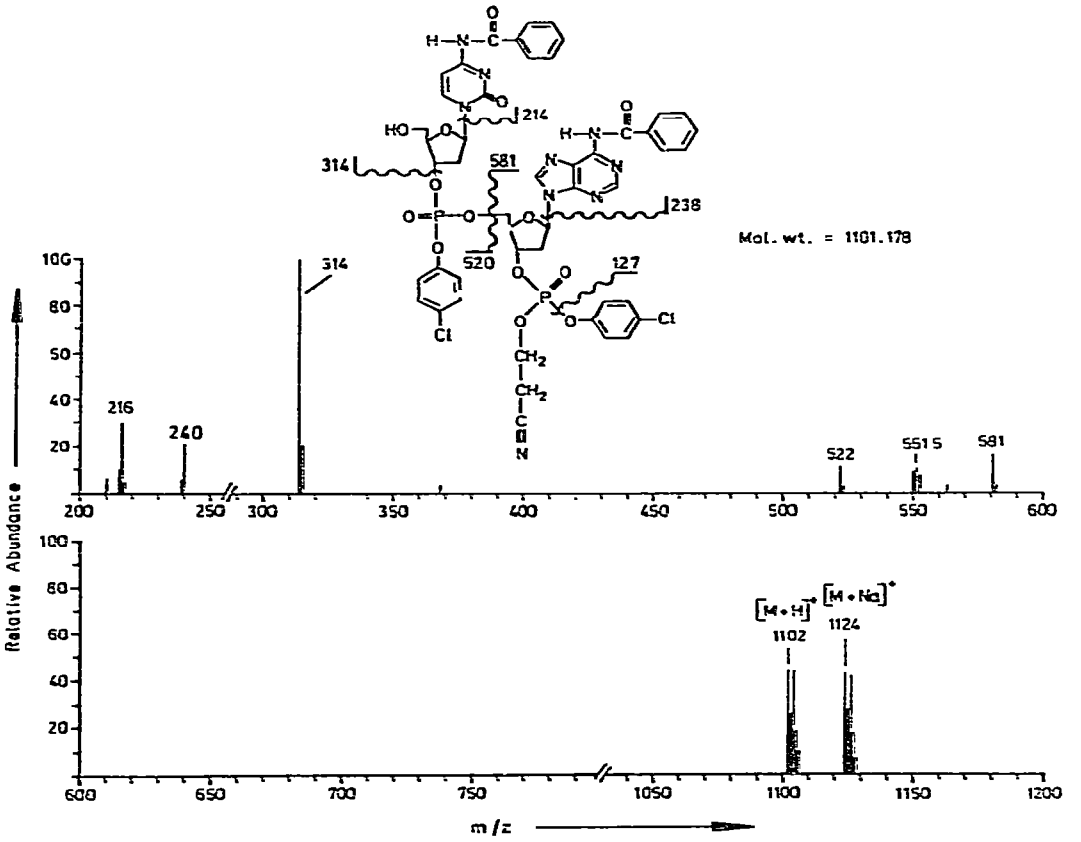

Fig. 13. FD mass spectrum of a partially protected, functionalized dinucleotide after HPLC separation[9]. Average of eight magnetic scans taken by the data system. The emitter heating current was 20 mA.

deblocked in the same way as 1 yielding, on further combination, longer oligonucleotide chains of defined sequence[55,56].

As the purity of the isolated intermediates greatly influences the success of further condensation steps, these products are routinely checked by TLC or by HPLC in a fast and efficient manner. Seliger *et al.*[10] have shown that FD-MS can be used as an additional and independent method for synthesis control of monomeric and dimeric building blocks.

Conventional EI-MS is not suitable for the identification of the nucleotides, because no molecular ions can be detected from these compounds owing to their low stability[57]. With FD-MS, at low temperatures, only protonated and cationized molecular ions of the nucleotides are produced, which allow the unambiguous determination of the molecular weights.

Fig. 12 shows the FD mass spectrum of a partially protected, functionalized mononucleotide after HPLC separation. The spectrum was obtained at an emitter heating current between 20 and 22 mA. At this temperature, in addition to the protonated molecular ion $[M + H]^+$ at m/z 486 and the cationized molecular ion [M

+ Na]$^+$ at m/z 508, fragment ions are produced that give structural information. Proton attack on the 3′-phosphoric acid ester bond yields *p*-chlorophenyl-β-cyanoethylphosphoric acid. Subsequent protonation of this neutral fragment on the emitter surface produces the signal at m/z 261 showing the characteristic isotope pattern of an organic compound containing one chlorine atom. Complementary to the protected phosphate moiety at m/z 261, the signal at m/z 224 can be assigned to the dehydrated nucleoside 2′,3′-dideoxy-2′,3′-dehydropyrimidineriboside and at m/z 225 to its protonated analogue. Cleavage of the N-glycosidic bond leads to the molecular ion of the unprotected base, appearing at m/z 126. The signal at m/z 128 is due to the *p*-chlorophenyl moiety, which again shows the characteristic isotope pattern for chlorine. The fragment at m/z 98 corresponds, according to its nominal mass, to a degradation product of deoxyribose found in the high-resolution FD mass spectrum of DNA[58]. Its structure was identified by collisional activation mass spectrometry as a mixture of α-angelica lactone and furfuryl alcohol[59].

Fig. 13 shows the FD mass spectrum of a partially protected, functionalized deoxyribodinucleoside diphosphate. Also in this instance the very clear and simple spectrum permits reliable molecular weight determination from the molecular ions of high relative abundance at m/z 1102 for [M + H]$^+$ and at m/z 1124 for [M + Na]$^+$. In addition, this determination is supported by the doubly charged molecular ions [M]$^{2+}$ at m/z 550.5, [M + 2H]$^{2+}$ at m/z 551.5 and [M + H + Na]$^{2+}$ at m/z 562.5. The base peak of the spectrum is formed at m/z 314 by fission of the 3′-ester bond of the 5′-terminal nucleoside and release of a protonated 2′,3′-dideoxy-2′,3′-dehydroriboside. Both nucleobases can be observed as protonated species at m/z 216 and 240, respectively. One of the most important fragmentations is obtained by rupture of the 5′-ester bond and proton attachment to the phosphate moiety of the 5′-terminal nucleoside yielding the complementary fragments at m/z 522 and 581.

We assume that both fragment ions are formed via protonation of the neutral species produced on the emitter surface during the FD process. The neutral species of m/z 521 represents the deoxycytidine-3′-phosphate residue of the original molecule, whereas the neutral species of m/z 580 corresponds to 5′-deoxy-4′,5′-dehydrodeoxyadenosine-3′-phosphate formed after cleavage of the 5′-ester bond of the dinucleoside diphosphate. Finally, an ion of relatively low abundance at m/z 128, not shown in Fig. 13, represents the *p*-chlorophenyl moiety of the compound.

These results show that FD-MS is not only a suitable technique for molecular weight determinations of protected nucleotides but also generates significant structural details. In view of the capacity of the technique for mixture analysis and for the detection of even minor components, FD-MS appears to be a useful method for the purity analysis of synthesized products.

4.3. Molecular and elemental analysis of natural products

The introduction of laser-assisted FD-MS[60–63] has resulted in better sensitivity and accessibility of the entire temperature range between 50 and 3000°C. In a recent investigation of chlorophyl a[62], the sample was purified by HPLC, transferred to the FD emitter and desorbed by stepwise laser heating. The FD mass spectra obtained allowed first the determination of the molecular weight (Fig. 14a), second the elucidation of structural details (Fig. 14b) and third direct isotope analysis of the central metal cation, with a precision of $< \pm 0.1\,\%$ (Fig. 14c). All three pieces of

information were obtained from the same microgram sample. This again demonstrates the unmatched versatility of the FD technique as an analytical tool for qualitative and quantitative analysis, elemental and molecular identification and determination of organic, organometallic and inorganic compounds.

5. CONCLUSION

A practical on-line coupling of HPLC and FD-MS has not yet been achieved and it is reasonable to assume that the additional technical complexity of such a coupling interface would make the FD part inefficient. The examples described above

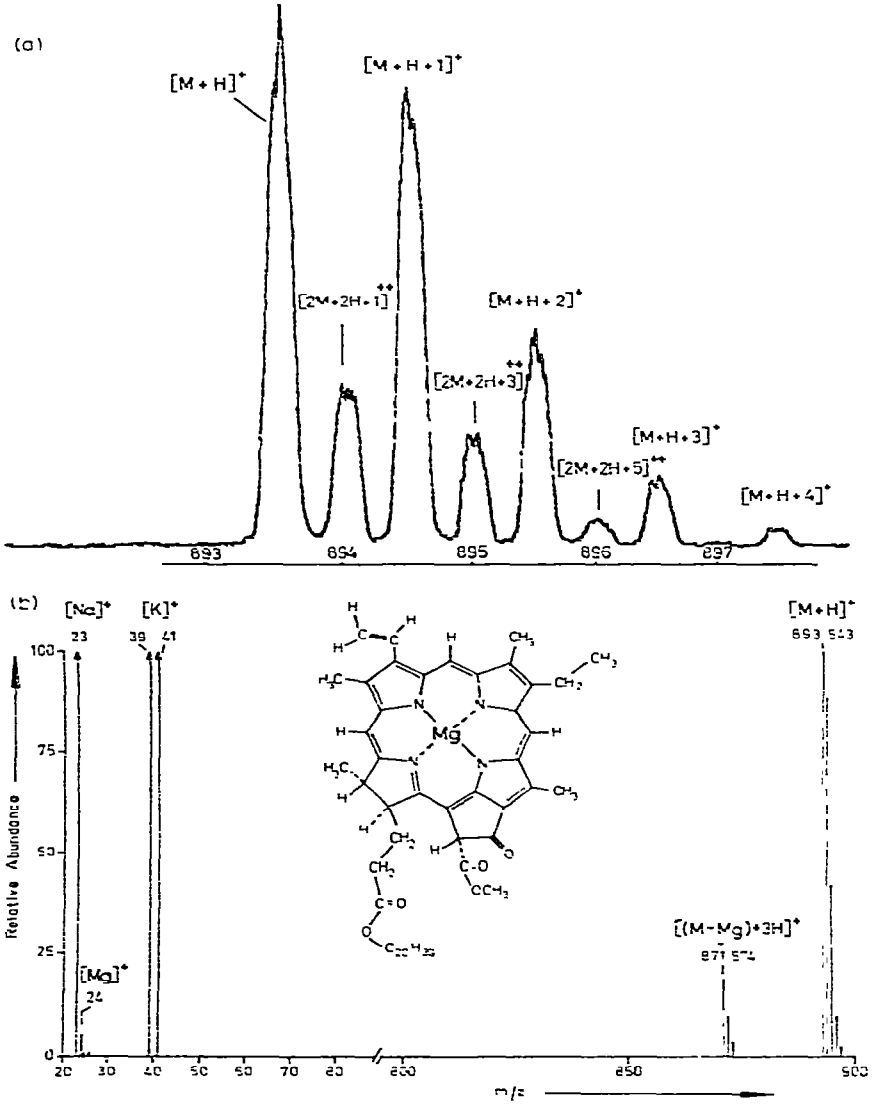

Fig. 14.

(Continued on p. 126)

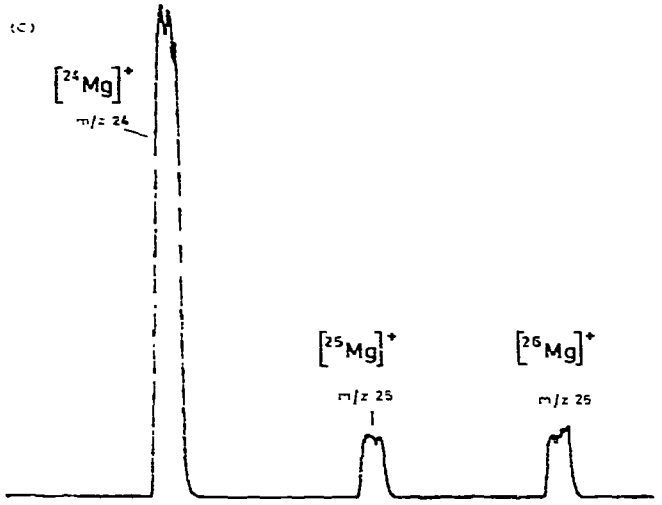

Fig. 14. Laser-assisted FDMS of chlorophyll a with a laser power of (a) 30 mW, molecular weight determination; (b) 30–0.5 W, averaged FD mass spectrum (50 magnetic scans); and (c) 1–2 W, recording and direct isotope determination of Mg isotopes with a multi-channel analyser[62].

show that this combination can be used in the off-line mode for solving a variety of problems. For investigations of medical, pharmaceutical and environmental samples, which often contain polar and thermally labile substances, the high selectivity of HPLC and the sensitivity of FD-MS are complementary; on the one hand, FD-MS serves as a detector for HPLC if the commonly used detectors are not sensitive enough, whereas on the other hand, HPLC can be used for purification of the sample if the FD-MS mass spectra are unclear. However, both methods are frequently used for the unambiguous confirmation of results. Further advantages of this combination are the small amounts of sample required (micrograms or less) and the short time required for one analysis. An HPLC separation normally requires between 10 and 30 min and the transfer of the eluents to the FD emitter takes only a few minutes, as does the FD analysis.

If the demands of the chromatography side on mass spectrometry are the availability of a wide mass range (up to mass 4000 and above[64]), high mass resolution ($> 10{,}000$) and the production of qualitative and quantitative data, the time factor for the analysis of the eluent becomes crucial. In this respect, the off-line method has an advantage as the different mass spectrometric investigations can be performed without time pressure. Further, a choice of different ionization methods is available. Within this framework, the use of quadrupole and time-of-flight instruments is unfavourable, but that of magnetic instruments is strongly advocated.

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7. SUMMARY

Field desorption mass spectrometry (FD-MS), which is applicable to analyses of compounds of low volatility and thermal instability, is used as a sensitive, specific and fast detection method for high-performance liquid chromatography (HPLC). Owing to its simplicity and efficiency, off-line combination is the preferred technique for the analysis of substances in chromatographic effluents. The principles of identification, purity control and quantification are presented. Characteristic examples of analytical investigations using the combination of HPLC and FD-MS in environmental, medical and pharmaceutical areas are given. Qualitative and quantitative results for drugs and endogenous compounds, *e.g.*, tranquillizers, immunosuppressive and antitumour agents and free amino acids, in human body fluids, biocides, *e.g.*, phenylureas, carbamates, organophosphorus and organometallic compounds, in river water, and natural and synthetic products, *e.g.*, saponins, chlorophyll and deoxyribonucleotides, are presented.

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