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Mass spectrometric techniques as aids in the diagnosis of gangrene in potatoes caused by Phoma exigua var. foveata

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Abstract

Current diagnosis of gangrene in potato tubers, caused by *Phoma exigua* var. *foveata,* is based on isolation and identification of the causal organism or on thin-layer chromatographic (TLC) detection of the anthraquinone pigment pachybasin (1-hydroxy-3-methylanthraquinone). In this paper alternative diagnostic approaches are described based on detection of specific ions by pyrolysis mass spectrometry (Py-MS), direct probe mass spectrometry (DIP-MS) and gas chromatography-mass spectrometry (GC-MS). The advantages and disadvantages of these methods are discussed in comparison with the TLC approach. GC-MS is a promising technique because of its high specificity, sensitivity and suitability for automation. Moreover, the MS approaches may lead to a reduction of total expertise time.

Additional keywords: Phytopathology, crop protection, pachybasin.

Introduction

Gangrene is an economically important disease of potato tubers; it is a rot which develops during storage as a result of damage at harvest. The disease is caused by the fungus *Phoma exigua* Desm. var. *foveata* (Foister) Boerema. It is presently restricted to Northern Europe, Australia and the high Andes in South America, and threatens the export of seed potatoes. The morphologically indistinguishable variety *P. exigua* var. *exigua,* is almost cosmopolitan in distribution but less serious as a pathogen of potatoes. Detailed descriptions of the disease are given by Boerema (1967, 1977) and Logan (1981). In the Netherlands, the discrimination between varieties is currently based on the TLC detection of the anthraquinone pigment pachybasin (1-hydroxy-3-methylanthraquinone) in extracts of affected potato tubers (Mosch and Mooi, 1975). The outcome of the diagnostic tests are decisive for certification and official granting of export licences.

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In the last decade mass spectrometric techniques have gained importance in routine biomedical research because of their sensitivity, specificity and microprocessor-based operation (Waller and Dermer, 1980; Gilbert and Self, 1981). The present paper describes the possibilities of mass spectrometric techniques as alternative aids in the diagnosis of gangrene in potatoes, using different types of sample introduction.

Gas-liquid chromatography (GLC) has been used to separate and characterize naturally occurring anthraquinones (Van Eijk and Roeijmans, 1976). Recently, the separation and characterization of these constituents was further improved using fused silica capillary columns and mass spectrometry (GC-MS) (Van Eijk and Roeijmans, 1984).

Other approaches are pyrolysis mass spectrometry (Py-MS) and direct insertion probe mass spectrometry (DIP-MS).

Materials and methods

Samples. Reference pachybasin was isolated from *P. exigua* var. *foveata* isolate 44. Pachybasin was also synthesized as described by Waldmann and Sellner (1938). The following potato isolates were studied: *P. exigua* var. *exigua* 2, 3, 10 and 11, and *P. exigua* var. *foveata* 13, 44, 49 and 50. All isolates had been obtained from the Research Institute for Plant Protection (IPO), Wageningen, the Netherlands. Whole potato tubers were superficially inoculated with a drop of a spore suspension and incubated for 1, 2, 3, 4 and 5 weeks at 5 °C. Tuber samples (500 g) were extracted twice with ethanol using a Waring blendor. Both extracts were combined and concentrated in vacuo to 200 ml. The extract was diluted with 300 ml water, acidified with 5M HCl to pH 3 and extracted twice with 200 ml ethyl acetate in a separatory funnel. The ethyl acetate solutions were evaporated to dryness in vacuo. The residues were redissolved in 1 ml chloroform/methanol (2 : 1, v/v). Aliquots of 1-2 µl of these solutions were used for TLC and GC-MS analyses. Pachybasin was analyzed in the underivatized state.

GC-MS. Analyses were performed with a Hewlett-Packard HP 5993B GC-MS system equipped with a splitless capillary column injection system and an OV-101 WCOT fused silica capillary column (12m, 0.2 mm ID). Operating conditions were: sample volume 1 µl, flow 2 ml helium/min^{-1} (pressure 69 kPa); linear oven temperature programming from 60 °C up to 210 °C at 5 °C/min, after an initial isothermal period of 1 min; 70 eV electron impact (EI) ionization; mass scan m/e 50 to 250, scan speed 325 amu/s. Selected ion monitoring (SIM) analyses were performed on mass 238.1 with 100 msec dwell time; temperature programming from 60 °C up to 210 °C at 30 °C/min, after an initial isothermal period of 1 min. SIM analyses were also run on a simplified mass analyzer HP 5970A interfaced with a HP 5880A gas chromatograph (Weijman et al., 1984).

Py-MS and DIP-MS. Samples for Py-MS and DIP-MS were prepared by suspending the freeze-dried and powdered material in methanol. Suspensions were applied to the probes and the solvent evaporated.

A description of the instrument and techniques involved in Py-MS have recently been given by Meuzelaar et al. (1982). Basically, the Py-MS system consists of a Curie-

point pyrolysis reactor mounted directly in the vacuum of a quadrupole mass spectrometer. The Curie-point technique implies rapid inductive heating of a Fe-Ni wire, stabilizing within 0.1 sec at 510 °C. Each sample was analyzed in duplicate. The spectra of the various samples were compared by multivariate analysis using the set of 20 most characteristic peaks (i.e. peaks with the highest ratio between sample variance/mean within sample variance, the Fisher ratio). Two-dimensional non-linear mapping of the resulting multidimensional relationships was performed by the procedure of Kruskal (Eshuis et al., 1977).

For DIP-MS analyses a Finnigan Model 3000 quadrupole mass spectrometer was used to record mass spectra from samples introduced directly into the ion source. Probe temperature was equal to the temperature of the ion source (about 50 °C). Spectra were recorded under 70 eV EI conditions.

Crude samples (cells, extracts), directly introduced into the vacuum of the mass spectrometer; can be heated up to a stable high temperature level (Py-MS) or heated to moderate temperature levels (DIP-MS). In Py-MS the sample is fragmented according to thermochemical principles and the volatile fragments formed are characterized by mass spectrometry under mild ionization conditions (14 eV electron impact). The resulting mass spectrum (pyrogram), largely representing molecular ions of pyrolytic fragments, can serve as a fingerprint reflecting the overall chemical composition of the sample. For diagnostic purposes, similarities between pyrograms can be calculated using multivariate techniques (Meuzelaar et al., 1982). In the direct insertion probe mode (DIP-MS) the sample is slowly heated close to the ion source of the massspectrometer to allow sublimation and ionization of volatile sample components.

TLC. Crude extracts and pachybasin reference were analyzed on thin-layer chromatography plates (Merck silicagel 60 F^{254}, thickness 0.2 mm) using the solvent system toluene/dioxane/acetic acid (90:25:4, v/v/v). Developed plates were inspected visually and in long wave UV light. With the TLC method reference pachybasin can be detected, under long wave UV light, down to a detection level of 50 ng (Weijman et al., 1984).

Results

Py-MS. Typical Py-MS fingerprints of potato tuber tissue infected with *P. exigua* var. *exigua* and *P. exigua* var. *foveata* are presented in Fig. 1. Fungal components contribute insignificantly to the total mass of the tubers. Consequently, the small quantitative differences between these spectra can be explained by the variable constitution of potato tubers, partly caused by reactions following infection (e.g. production of phytoalexins or enzymes). Among the most characteristic peaks underlying the differences presented in Fig. 1 A, B and Fig. 2 (including data from 16 spectra) were m/e 94, 108, 120, 122, 136 and 150, which are indicative of phenolic constituents (Meuzelaar et al., 1982). The intensities of these peaks were higher in potato tissue infected with var. *foveata.* The phenolic peaks can be used as single parameters for screening of infections with var. *foveata.*

The multidimensional relationships of peaks resulting from computerized multivariate analysis are presented graphically in the form of a non-linear map (Fig. 2). This map clearly illustrates the ability of the Py-MS method to discriminate be-

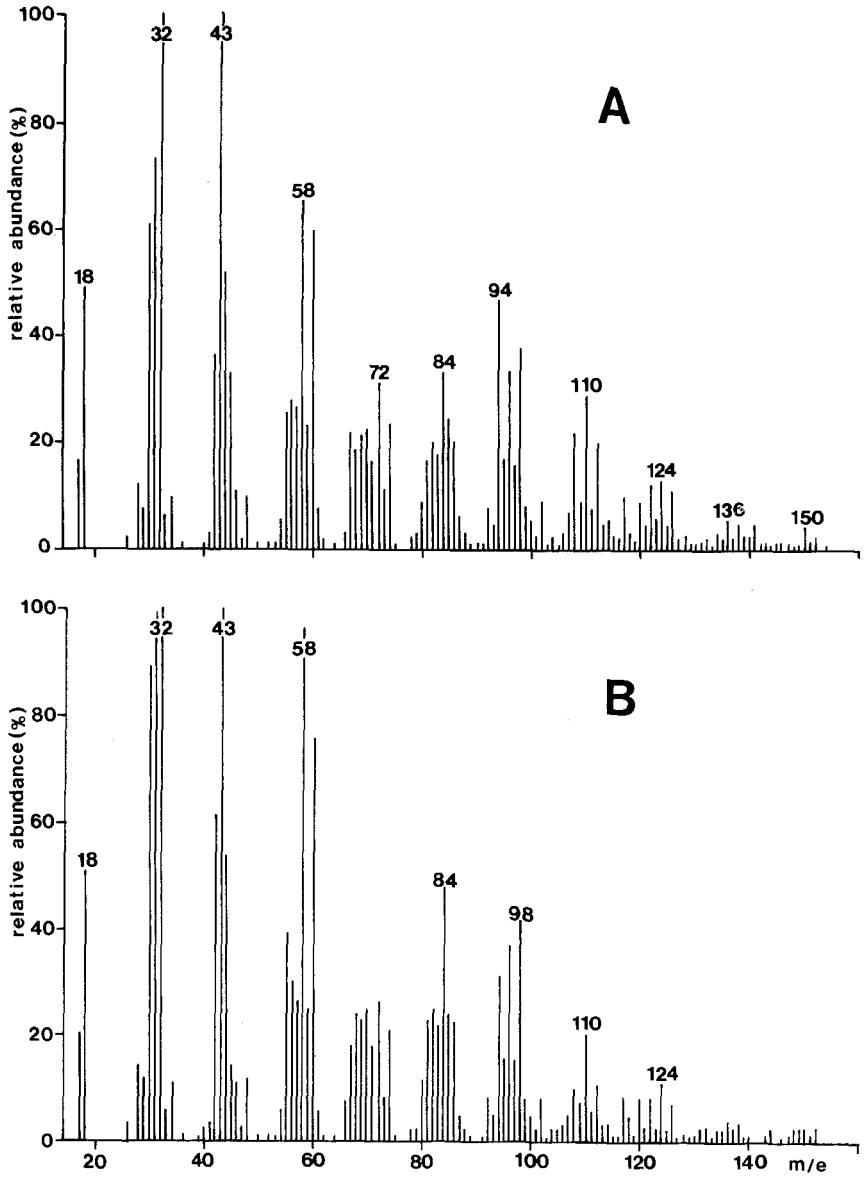

Fig. 1. Pyrolysis mass spectra of *Phoma exigua* var. *foveata* isolate 44 (A) and *Phoma exigua* var. *exigua* isolate 11 (B).

tween infections by the two *P. exigua* varieties.

Reproducibility, a crucial factor for diagnostic purposes, can simply be judged from the distances between duplicate spectrum points with respect to the scattering observed between different samples.

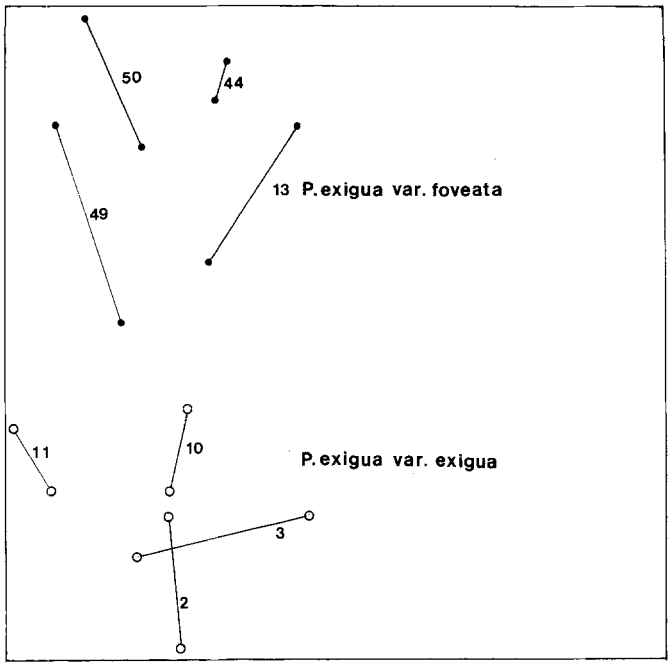

Fig. 2. Non-linear map representing multidimensional relationships between pyrolysis mass spectra of eight preparations of potato tissue infected with *Phoma exigua* var. *foveata* (●) and of tissue infected with *Phoma exigua* var. *exigua* (○). Each point represents a spectrum, duplicate spectra are connected. Isolate numbers are indicated.

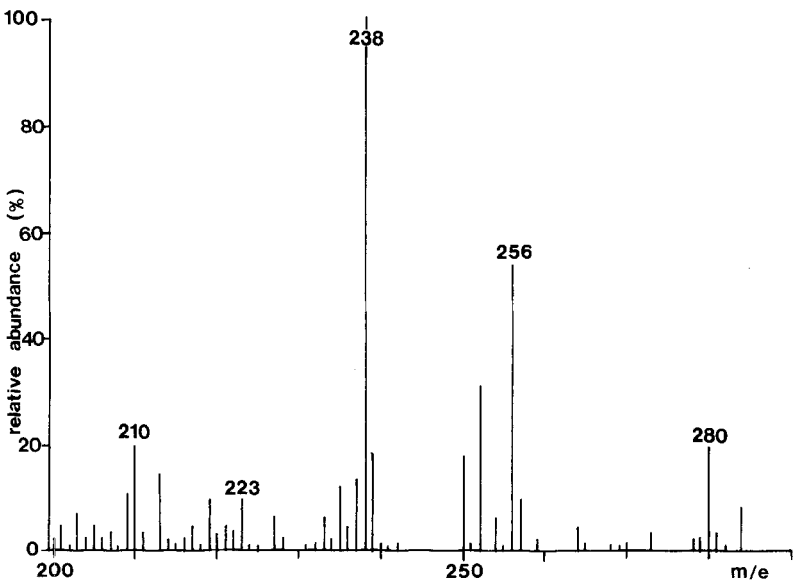

Fig. 3. Mass spectrum, using a direct insertion probe, of potato tissue experimentally infected with *Phoma exigua* var. *foveata* isolate 44.

DIP-MS. A typical mass spectrum, recorded after direct introduction of potato tissue experimentally infected with *P. exigua* var. *foveata,* is presented in Fig. 3. The simplicity of the spectrum resulting from this complex sample illustrates the selective liberation of volatile components under moderate temperature probe conditions. The dominant peak at m/e 238 reflects the presence of pachybasin (see Fig. 4) among other components of unidentified nature. As judged from the very low intensity of the peak at m/e 238 pachybasin is absent or present in negligible amounts in samples of potato tissue infected by var. *exigua.* The ion at m/e 238 in DIP mass spectra can be used as the target ion for diagnostic purposes.

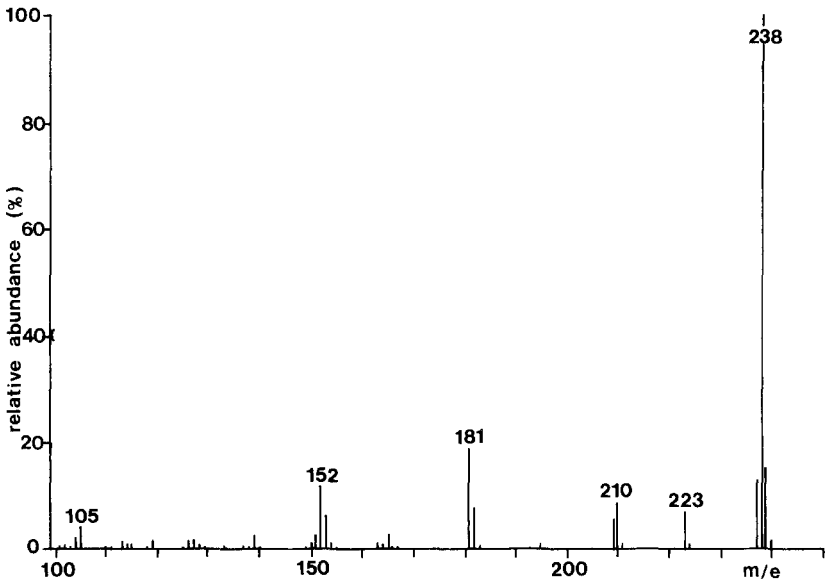

Fig. 4. Mass spectrum of pachybasin reference (70 eV EI).

GC-MS and TLC. The GC-MS approach is based on the stability of pachybasin under 70 eV EI conditions, reflected by the dominance of the molecular ion of pachybasin at m/e 238 in the mass spectrum (Fig. 4). Detection of this ion in the SIM mode of operation leads to a detection limit of about 50 pg, without special tuning of the mass spectrometer. The specificity of GC-MS is illustrated in Fig. 5 and is indicated by a single peak in the mass chromatogram of m/e 238.

To compare the diagnostic possibilities of both GC-MS and TLC, 1 μl of the same sample was analyzed by both methods. The mass spectrometer was operated in the SIM mode. The results are presented in Table 1.

Pachybasin could not be detected in incubated control potato tissue, nor in potatoes experimentally infected with other potato pathogens developing rots: *Fusarium solani* var. *coeruleum, F. sulphureum,* bacteria or *P. exigua* var. *exigua.*

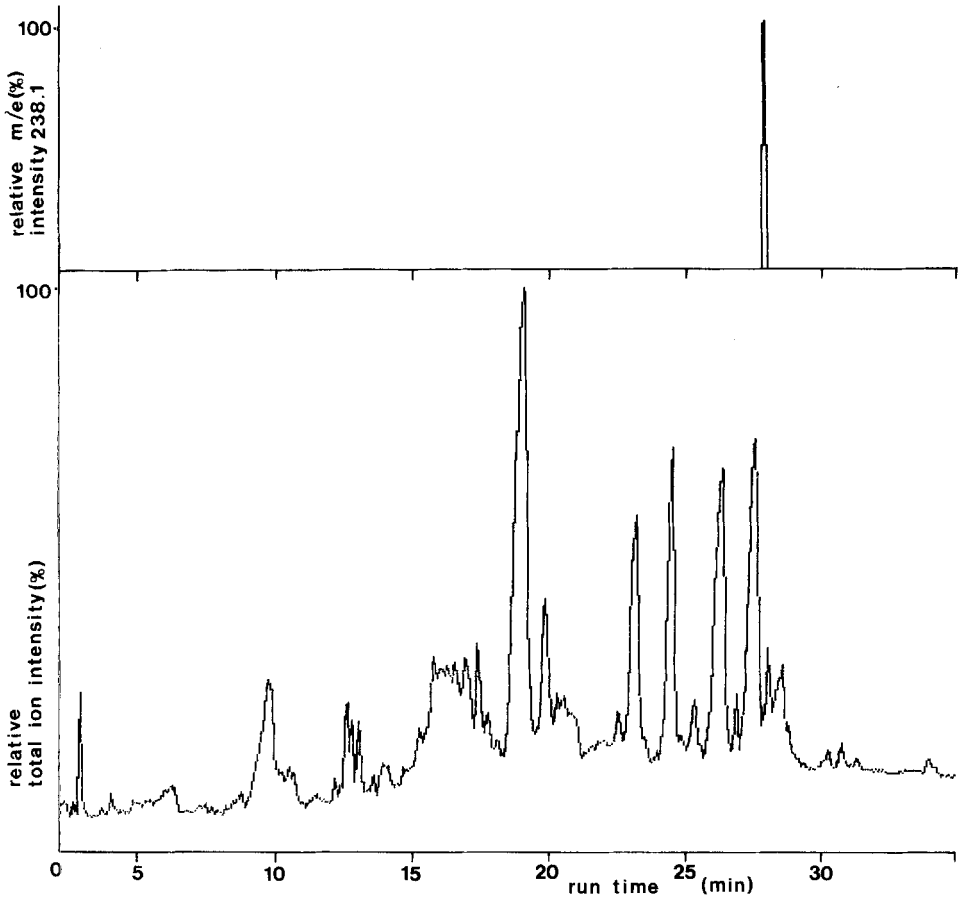

Fig. 5. Total ion gas chromatogram of an extract of potato tissue experimentally infected with *Phoma exigua* var. *foveata* isolate 44 and mass chromatogram of m/e 238.1.

Table 1. Detection of pachybasin in extracts of potato tubers infected by a mixture of *Phoma exigua* var. *foveata* isolates.

Incubation period (weeks)	GC-MS-SIM (counts/g)	Gangrene diagnosis	
		by GC-MS-SIM	by TLC
1	0.4	–	–
2	8.8	+	–
3	44.3	+	–
4	201.8	+	+
5	1054.1	+	+

Discussion

TLC is still a frequently used technique to detect gangrene because of its low cost and simplicity (Mosch and Mooi, 1975). However, the development of pachybasin spots is often disturbed because of limited separation power when analyzing crude extracts.

The MS methods studied as alternative approaches, although more expensive, are faster and more reliable. Gangrene caused by *F. exigua* var. *foveata* can now be identified in an early stage of development. Identification in DIP-MS and in GC-MS is based on fully resolved specific ion peaks (Waller and Dermer, 1980). A disadvantage of the DIP-MS method for routine use is the necessity of frequent ion source cleaning due to contamination of the system by many components of low volatility. This problem can be avoided when only components of interest, isolated by a gas chromatograph, are introduced in the ion source (GC-MS). Py-MS is a non-target approach for fast discrimination of biological samples (Meuzelaar et al., 1982). Discrimination is based on a set of statistically selected, but unidentified, ions formed after pyrolysis and ionization. A priory selection of a diagnostic component is not required. Nevertheless, biochemical interpretation in general terms is possible to some extent, by judging sets of covariant mass peak intensities. Thus differences in proteins, phenolics, polysaccharides etc. can be indicated. In the case of gangrene, discrimination seems to be based on phenolic substances which are produced by the host tissue as part of the defense mechanism. The production of phenolics was also documented in detail by Glazener (1982) for tomato fruits infected with *Botrytis cinerea.* Recently, similar observations have been made by Windig et al. (1983) for virus infected plant material. In theory, these phenolic response products can be detected early after infection long before the fungus extensively invades the host and produces detectable levels of pachybasin. Py-MS is a tool of interest to monitor and study unidentified interactions in plant pathogenesis. Modern routine Py-MS instruments can be run unattended with an analysis capacity of 30 samples per hour. Commercial instruments have become available recently.

To date, GC-MS is the promising method for routine diagnostic application because of general availability, specificity and suitability for automation. Recently, a simplified mass spectrometric detector for use with existing gas chromatographs has been introduced. This system is designed for routine diagnostic purposes (Weijman et al., 1984). Using GC-MS techniques in the case of gangrene diagnosis, the detection level of pachybasin can be brought down to the picogram range in the SIM mode of operation. Without special tuning, GC-MS is a factor of 1000 more sensitive than TLC.

For routine application GC-MS in the SIM mode can be further optimized by maximizing the transfer of pachybasin to the ion source. This can be achieved by increasing the concentration of pachybasin in the samples and by applying a short packed column allowing increased sample loading. To further increase sensitivity the tuning parameters of the mass spectrometer must be adjusted to obtain maximum abundance at m/e 238. Analysis time can be dratically reduced since the separation step is not crucial when monitoring at m/e 238 (Fig. 5). The modern computerized systems enable automatic sampling, analysis, dataprocessing and report generation. Quantification can be improved using deuterated pachybasin as an internal standard. Optimization of analysis parameters will ultimately lead to an increase of sample analysis capacity and to a reduction of total expertise time.

Samenvatting

Massaspectrometrische technieken als hulp bij de diagnose van gangreen in aardappels veroorzaakt door Phoma exigua var. foveata

De huidige diagnostiek van gangreen in aardappelknollen, veroorzaakt door *Phoma exigua* var. *foveata*, is gebaseerd op de isolatie en identifikatie van de schimmel of op de dunnelaag-chromatografische (TLC) bepaling van het pigment pachybasine (1-hydroxy-3-methylanthrachinon).

In dit artikel worden nieuwe diagnostische benaderingen beschreven die gebaseerd zijn op de detectie van specifieke ionen door middel van pyrolyse-massaspectrometrie (Py-MS), direct-probe-massaspectrometrie (DIP-MS) en gaschromatografie-massaspectrometrie (GC-MS). De voor- en nadelen van deze methoden worden behandeld en vergeleken met de dunnelaag-chromatografische methode.

GC-MS is een veelbelovende methode vanwege de grote specificiteit, gevoeligheid en geschiktheid voor automatisering. Op den duur kunnen de MS-methoden leiden tot een verkorting van de totale expertisetijd.

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