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Pyrolysis in Biotechnology

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Introduction

Pyrolysis is the thermal decomposition of molecules in an inert atmosphere. The transfer of thermal energy to a polymeric network or to macromolecules causes degradation of the sample into volatile products. The reaction products are characteristic of the original structure and much more easily analysed than the original sample. A significant advantage of the pyrolysis technique is the speed of the analysis. Complex materials that normally require time consuming analysis can be investigated by this technique coupled to gas chromatography (Py-GC) in less than an hour or with it coupled directly to a mass spectrometer (Py-MS) in a couple of minutes.

C.G. Williams' article from 1862 is considered to be the first of its kind in the field of pyrolysis. That study identified isoprene as the main pyrolytic product of rubber. However, broad use of analytical pyrolysis has had to wait for the development of modern analytical technology. Today, pyrolysis is widely used to study macromolecules, including synthetic and natural polymers (see for example Wampler, 1989), to perform degradation and kinetic studies and also for the qualitative and quantitative analysis of complex substances. Examples of material analysed by pyrolysis are synthetic polymers (for a review, see Blazsó, 1997), coating materials (Haken, 1999), rubber (Dubey *et al.*, 1995), paper and paper coating, plant material (Ralph and Hatfield, 1991) and bacteria. Pyrolysis is also used in forensic science, art and archaeology (Shedrinsky *et al.*, 1989).

This present review focuses on the analytical pyrolysis of biological macromolecules such as proteins, DNA and microorganisms. The first part of the article presents an overview of the pyrolysis techniques available and the methods for analysis of the pyrolytic products, the pyrolysate. The second part presents some applications to illustrate the types of problem that researchers have been able to solve using pyrolysis.

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The pyrolytic process

AN OVERVIEW AND SOME DEFINITIONS

The process of analytical pyrolysis includes:

- introduction of the sample (liquid or solid)
- the pyrolysis process itself
- transfer of the pyrolysate to an analytical instrument for analysis
- and finally, analysis of the data produced

Since degradation is to be caused only by thermal energy, pyrolysis is performed in an inert atmosphere, usually consisting of helium or nitrogen. The gas also transports the pyrolysate away from the region of high temperature, known as the 'hot zone'. Chemical reactions taking place between the pyrolytic products or catalytic effects due to contact between metal surfaces and the sample are called secondary reactions. These reactions are unwanted since they are not as reproducible as the initial degradation process.

The temperature increase during pyrolysis can be divided into three steps: the initial temperature, the rising temperature and the final temperature. A graphical representation of temperature versus time is called a temperature time profile, TTP. A typical example is shown in *Figure 1.1*. The total heating time, THT, is the time from the start to the end of the sample heating in a pyrolysis experiment. The temperature rise time, TRT, is the time required for the pyrolysis temperature to increase from its initial to its final temperature. The final (steady state) temperature attained by the pyrolyser is referred to as the final pyrolysis temperature, $T_{(f.Py)}$. Final pyrolysis temperatures are usually between 400 and 900°C. Degradation caused by temperatures below 300°C is usually called thermal decomposition and not pyrolysis.

Common pyrolysis techniques are isothermal, fractionated, stepwise and sequential pyrolysis. In isothermal pyrolysis the temperature is kept essentially constant at the final pyrolysis temperature. In fractionated pyrolysis the same sample is pyrolysed at different temperatures for different times in order to study special fractions of the sample. In stepwise pyrolysis the temperature is raised in steps and the pyrolysate produced at each step is analysed. Stepwise pyrolysis is usually used to study kinetics. Sequential is when the same sample is repetitively pyrolysed under identical conditions, i.e. final pyrolysis temperature, temperature rise time and total heating time, and the products are analysed after each pyrolysis (for an explanation of nomenclature, see Uden, 1995).

INSTRUMENTS USED FOR PYROLYSIS

Pyrolysers available on the market are constructed in basically the same way, with a control unit and a pyrolysis reactor. The controller provides the electricity needed for inducing the thermal degradation and the pyrolysis reactor provides the required environmental conditions for pyrolysis. The pyrolysis body (also called the 'interface') is the housing of the main heating element into which the sample is introduced before pyrolysis. The interface is often mounted on top of the normal injector of the analytical instrument. A flow of inert gas passes through the interface to transport the

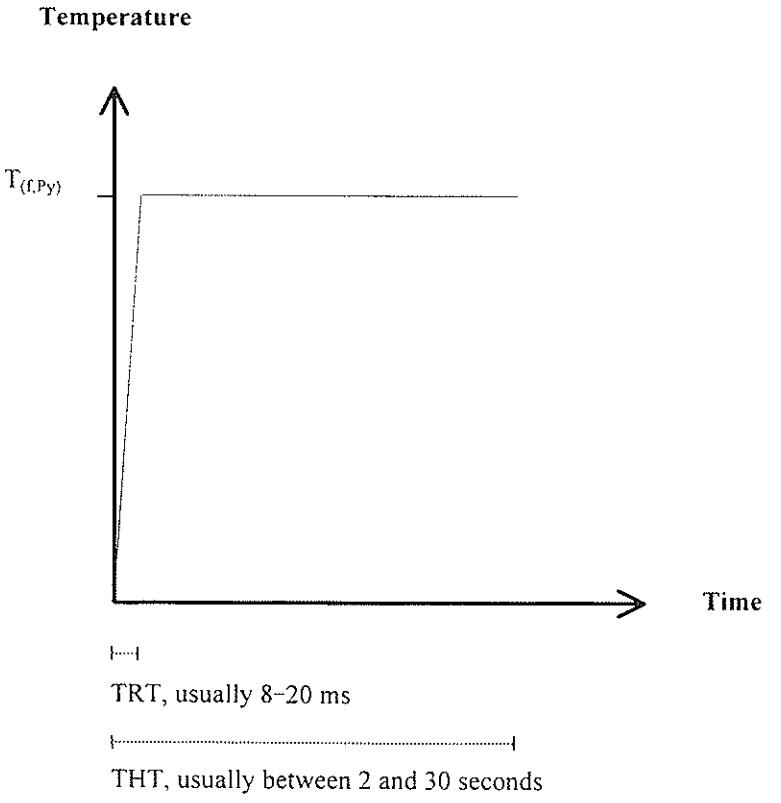


Figure 1.1. Illustration of time temperature profile, TTP, with temperature rise time, TRT, final pyrolysis temperature, $T_{(f,Py)}$, and the total heating time, THT, marked.

volatiles produced by the process into the analyser. The inner wall of the pyrolyser body is often covered with quartz glass to avoid secondary reactions of the substances caused by the catalytic effects of metal surfaces. To prevent condensation of high mass molecules, the pyrolyser body is usually heated to 150–300°C.

Three types of commercial pyrolysers are shown schematically in *Figure 1.2*. The design of the main heating element divides the pyrolysers into two classes: heated filament and furnace pyrolysers. In filament heated pyrolysers the sample is placed directly on a cold filament. The filament can have different shapes depending on whether the sample to be analysed is a solution, suspension or solid. The probe to which the filament is coupled is inserted into the pyrolyser body and the filament is heated to the required pyrolysis temperature by a controller unit. In furnace pyrolysers, also called continuous pyrolysers, the pyrolysis reactor is already set at the required temperature before the sample is introduced.

There are also radiative heated pyrolysers where a laser can be used for heating. However, to date, very little has been published on such pyrolysers. In *Table 1.1* the characteristics of the pyrolysers mentioned in the text above are compared.

Heated filament pyrolysers: inductively and resistively heated

There are two types of heated filament pyrolysers: inductively and resistively heated

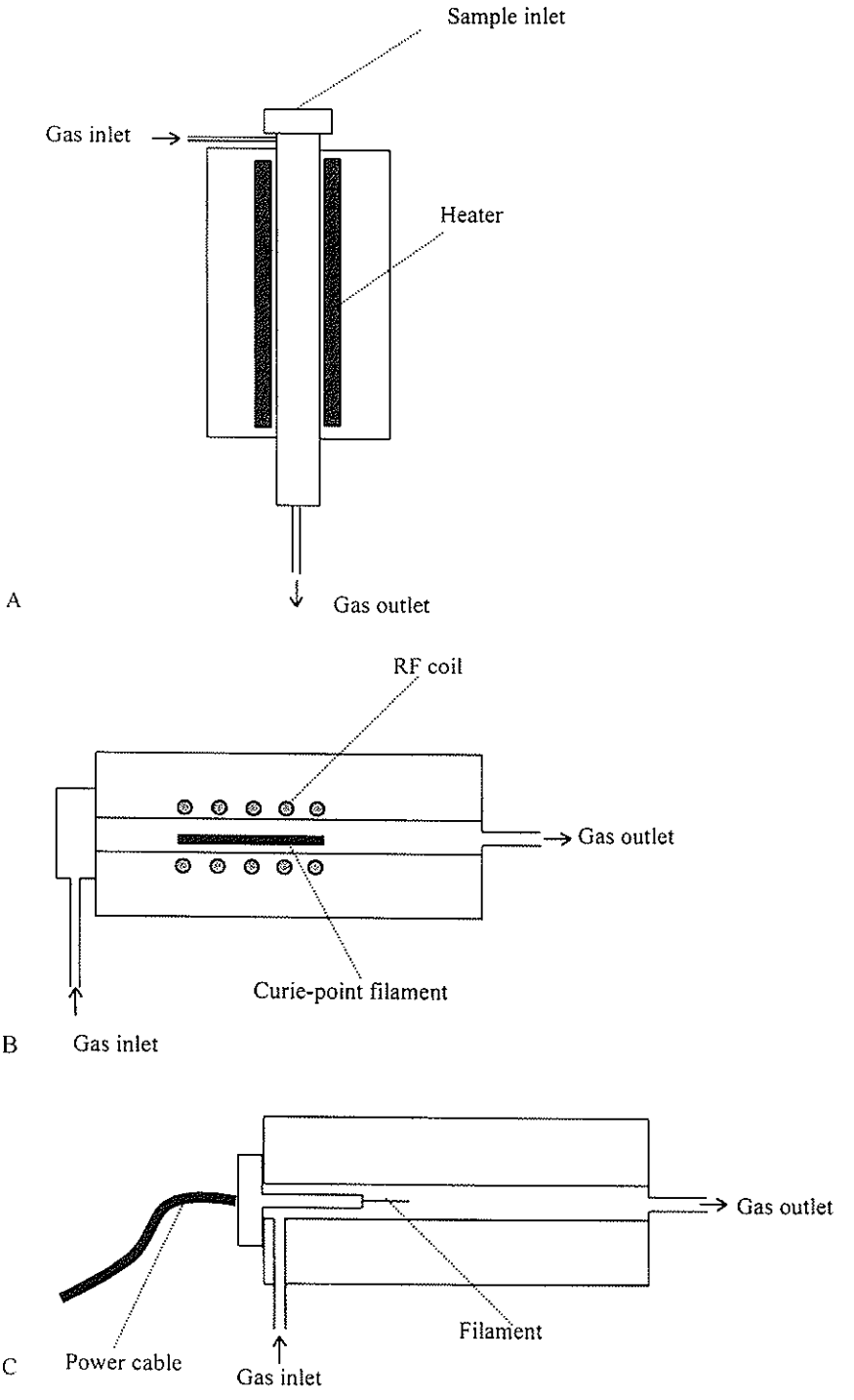


Figure 1.2. Schematic presentation of the three most common types of pyrolysers: continuous mode (A); inductively (B); and resistively (C) heated filament pyrolyser.

Table 1.1. Comparison of the characteristics of four types of pyrolysers

	Inductively heated	Resistively heated	Continuous mode	Radiative heated
Temp. choice	Discrete	Continuous	Continuous	Uncontrolled
Temp. limit °C	1128	1300	1500	Very high
Temp. gradient	Not possible	Possible	Possible	Possible
Stepwise/fractionated py	Not possible	Possible	Not possible	Not possible
Minimum TRT	70 ms	8 ms	0.2 s	10 μs
Reproducibility	Very good	Very good	Good	Not good
Catalytic reactions	Some	Low	Low	Very low

filament. Inductively heated pyrolysers utilize the principles of inductively heated furnaces. By heating a filament made from a ferromagnetic material, a simple and accurate temperature control can be achieved. The inductive coupling is dramatically reduced when the filament temperature passes the Curie temperature. Inductively heated pyrolysers are usually called Curie-point pyrolysers. To avoid catalytic effects, some suppliers offer gold-plated ferromagnetic holders. With a powerful control unit the Curie-point pyrolyser filament can be heated in milliseconds. The most obvious disadvantage of an inductively heated filament is the inability to freely choose the final temperature. Another disadvantage is the lack of possibility to perform stepwise and fractionated pyrolysis, since different temperatures require different alloys in the filament. The Curie-point pyrolysers are best suited for liquids or soluble materials that can be coated onto the filament in a thin layer. Solid samples that can be melted onto the filament surface can also easily be analysed.

Similar to the Curie-point pyrolysers, the resistively heated pyrolysers operate by taking the filament from ambient to pyrolysis temperature in a very short time. In resistively heated pyrolysers, well-controlled electric pulses are used to control the temperature of the filament. Platinum is the material most commonly used in the filament. The major disadvantage of resistively heated pyrolysers is that the user has to calibrate the filament to be able to control the pyrolysis temperature. Another problem is the risk that the filament is unevenly heated throughout its length so that, in order to experience the same pyrolysis temperature, the sample has to be placed in the same spot of the filament every time (Tydin-Ericsson, 1973). The advantage with a resistively heated filament is the ability to freely choose the final pyrolysis temperature and the ability to perform stepwise or fractionated pyrolysis.

Continuous mode pyrolysis

In continuous mode pyrolysers the whole pyrolyser body is heated to the desired pyrolysis temperature. The sample is introduced into the hot zone when the pyrolysis temperature is reached. To achieve a short TRT the sample has to be introduced very rapidly into the furnace. Liquid samples or suspensions are easily introduced using a standard syringe. However, it can be difficult to avoid the introduction of air when introducing solid samples. Some suppliers have solved this problem by designing special tools for the introduction of solid samples. Since the pyrolyser is kept constantly at a high temperature, the pyrolysate has to be quickly swept away to avoid secondary reactions. This is achieved by a very high gas flow through the furnace,

commonly 100 ml/min. The greatest advantages of furnace pyrolysers are their simple design, ease of use, and the low cost.

ANALYTICAL TECHNIQUES USED WITH PYROLYSIS

Since pyrolysis results in a large number of different substances, the pyrolysate has to be transferred to an analytical instrument for analysis. There are two main transfer methods: on-line and off-line. In off-line pyrolysis the pyrolysate is dissolved in a solvent and can be saved for future analysis. Off-line transfer is less common than on-line, but can be used for obtaining information on the components of the pyrolysate that are difficult to transfer directly to an analytical instrument or for the analysis of materials associated with char. In on-line transfer the pyrolysate is usually swept from the heat source by the flow of inert gas into the analytical instrument.

Gas chromatography, GC, coupled to different types of detectors is a common technique utilized for the analysis of pyrolysates (reviewed by Wampler, 1999). GC is a well-established technique with an outstanding capacity to separate compounds depending on polarity and/or boiling point (Eiceman *et al.*, 2000). The obvious advantage of using mass spectroscopy (MS) as the detector is its capacity to identify unknown compounds. Infrared, IR, spectrometers and Fourier transform infrared, FTIR, in particular, have also been used as detectors in GC (Weber, 1991). Just as with MS, FTIR can quantify and identify the sample as a fingerprint. A disadvantage with FTIR is the lower sensitivity compared to MS, but it may give reproducible data more easily. FTIR is usually used where MS analysis is less successful, for example in polymer analysis when gases such as NH_3, CH_4 and CO_2 are formed.

Pyrolysis coupled directly to MS is also frequently used. This technique generates a complex spectrum in which the abundance of each mass is the sum of the contributions of all substances having charged fragments for that specific mass. Pyrograms from the analysis of a liquid chromatography media by Py-GC/MS and a Py-MS spectrum of the same sample are given in *Figure 1.3*.

Since large molecules in a pyrolysate usually give more structural information than small ones, liquid chromatography would appear to be a better alternative than GC for the analysis of the pyrolysate. However, since compound identification is difficult when using LC, even when coupled to MS, GC is still the most popular alternative (Arisz *et al.*, 1990). The different techniques using Py, GC and/or MS are compared in *Table 1.2*.

PARAMETERS INFLUENCING THE REPRODUCIBILITY OF THE PYROLYSIS PROCESS

The sample, the pyrolysis process and the transfer of pyrolysate have been pointed out as the three major sources of imprecision in the pyrolysis process (Wampler and Levy, 1987) and will now be considered.

Influence from the sample

The typical sample amounts in analytical pyrolysis vary from a few micrograms to a few milligrams.

Wolf and Levy showed in 1973 that large sample sizes lead to a longer TRT, as a

consequence of which the sample starts to decompose before reaching the final pyrolysis temperature. Large sample amounts also increase the risk of secondary reactions (Levy, 1967). Since the sample is to be heated as fast and evenly as possible, the optimal sample shape is a thin film. This is easy to accommodate with liquids but harder with suspended materials or solid samples. Another problem with solid samples is to get a homogeneous mixture. The homogeneity is also an important parameter as well as shape, specific heat and the heat transfer between the pyrolyser heat source and the sample (Wampler and Levy, 1987).

Influence of the heating process

The heating rate has been shown to be a very sensitive parameter for reproducibility (Farré-Ruis and Guiochon, 1968). The pyrolysers of today have a very fast TRT which, in most cases, is under control. In their article from 1972, R.L. Levy, D.L. Fanter and C.L. Wolf recommend the user to have a TRT of at least one magnitude shorter than half the decomposition time of the sample, as a general rule.

The final pyrolysis temperature is also important. Generally, the higher the final pyrolysis temperature the smaller and less characteristic fragments are produced. The choice of final pyrolysis temperature depends both on the material to be pyrolysed and on the purpose of the analysis.

Influence of the transfer of the pyrolysate to the analytical instrument

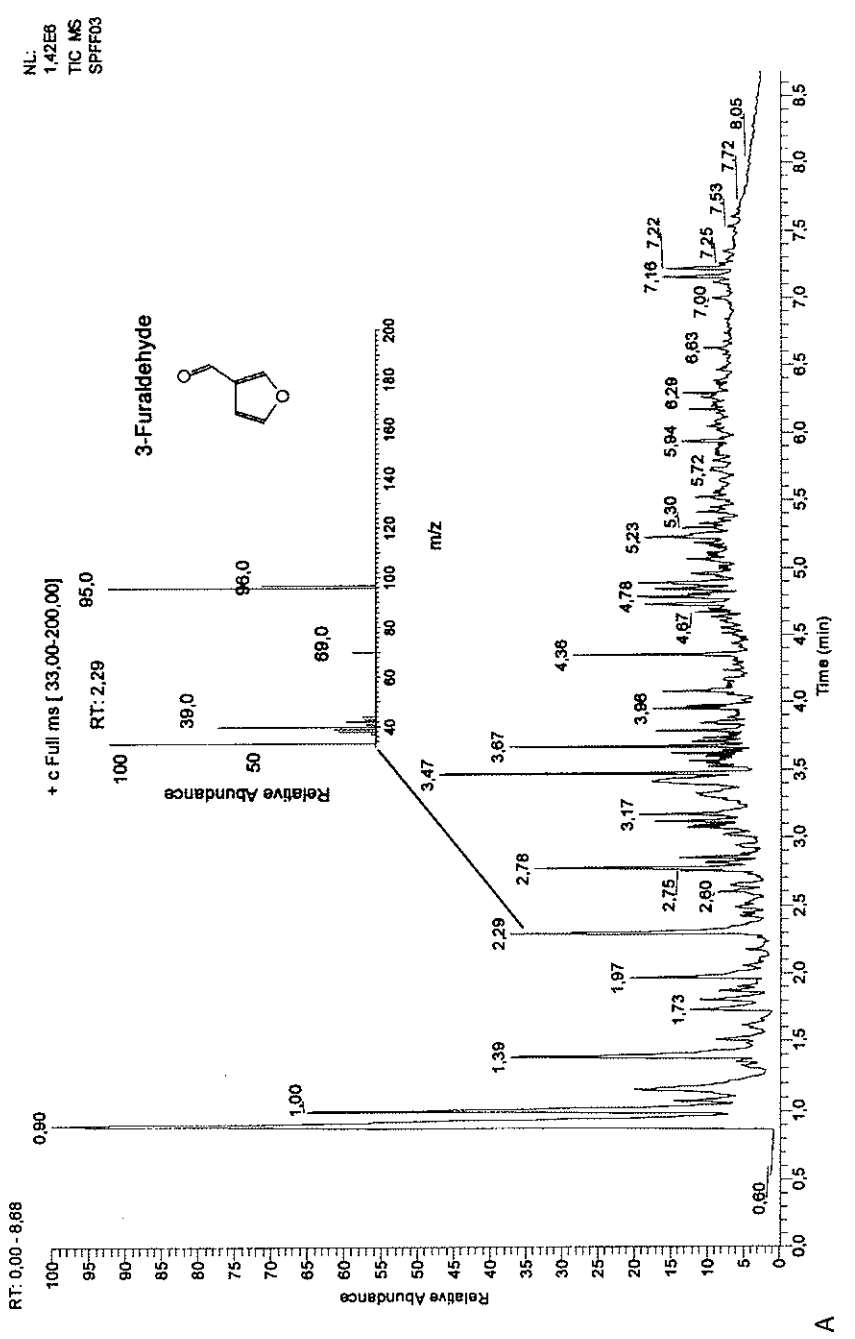
The longer the pyrolysate stays in the hot zone, the higher is the risk for secondary reactions. Setting a high flow rate through the pyrolyser (Levy, 1967) decreases the number of secondary reactions. A flow rate of 30–40 ml/min is commonly used (often higher for continuous mode pyrolysers). The reproducibility of the pyrolysis is also affected by condensation of high molecular weight molecules at the interface. To prevent condensation in heated filament pyrolysers, the interface is pre-heated, typically to 150–300°C. There is, however, usually a waiting time for the sample in the interface before pyrolysis, and there might be a risk of sample decomposition during this time.

In conclusion

It might seem difficult to have all these parameters in mind during method development. Luckily, the suppliers of the pyrolysers have already taken care of many of them, such as TRT and precise temperature control. The most important of these for the user to optimize is the sample amount and the final pyrolysis temperature. It is, however, worth noting that other factors, for example the sodium chloride content, can influence the result (Snyder *et al.*, 1988).

DATA EVALUATION

As has been mentioned above, data from Py-GC and Py-MS analysis are usually very complex. Studying the pyrogram as a fingerprint or analysing single peaks or fragments can give the required information but the use of multivariate data analysis


NL:
1,42E8
TIC MS
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RT: 0,00 - 8,68
Relative Abundance
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2,80
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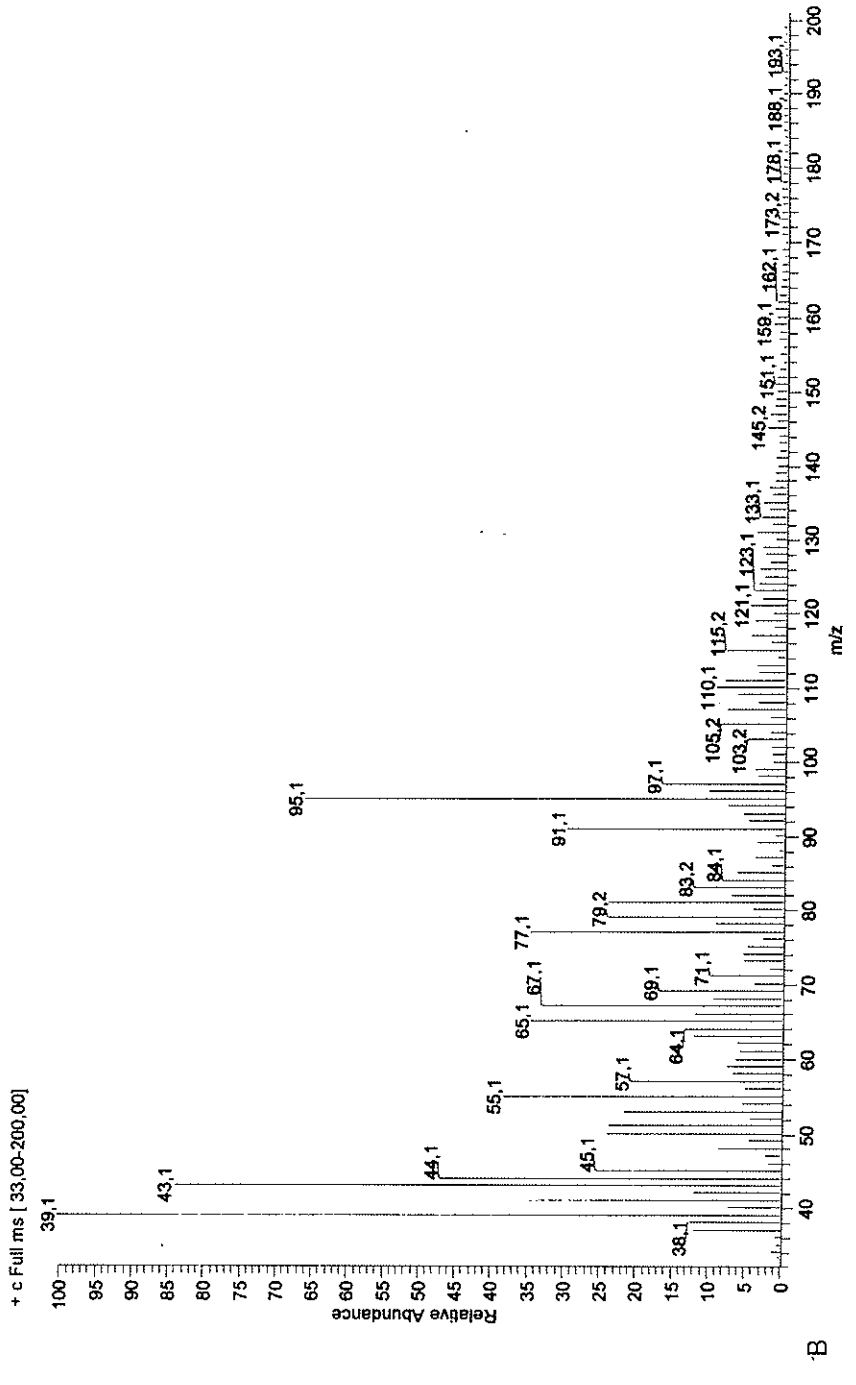

Figure 1.3. Pyrogram (A) and Py-MS spectrum (B) from pyrolysis of a liquid chromatography medium: SP Sepharose FF.

Table 1.2. Comparison between Py-GC, Py-MS and Py-GC/MS

	Py-GC	Py-MS	Py-GC/MS
Transfer efficiency	OK	Good	OK
Analysis time	Hours	Minutes	Hours
Reproducibility	Good	Good	Good
Multivariate reproducibility	Very good	Good	Good
Qualitative information	Possible with standards	Possible	Very good
Quantitative information	Good	Good together with multivariate analysis	Good

has greatly improved the possibilities for extracting information. This is particularly true when analysing microorganisms where classification is often the aim of the study: this type of analysis has in this case become common, but it is also applicable for quantification (Goodacre *et al.*, 1994b).

Since each point (mass-intensity or retention time) may have a multiple origin and some data points are of no use, the selection procedure of useful data is important. To address this, principal component analysis, PCA, and canonical variate analysis, CVA, are used (Adams, 1998). These procedures also maximize discrimination between the different sample classes (Goodfellow *et al.*, 1994).

Hierarchical cluster analysis is a useful technique to display the total discrimination within the data. All the variance is displayed in two dimensions in dendograms or minimum spanning trees (Gutteridge *et al.*, 1985). This approach of data handling has so far been applied mainly to Py-MS studies. Eigenvectors are used in order to generate a distance matrix that contains information on Mahlanobis distances.

The most recent approach for the processing of Py-MS results is the use of artificial neural networks (ANNs) (Goodacre and Kell, 1996). ANNs are a means of uncovering complex, non-linear relationships in multivariate data that can take numerical data and transform it into comprehensible output. The great power of neural networks stems from the fact that it is possible to 'train' them. One can acquire sets of multivariate data (e.g. pyrolysis mass spectra) from standard materials of known identities and train ANNs using these identities as the desired outputs (Zupan and Gasteiger, 1999). This combination of Py-MS and ANNs has now been employed for the identification of strains of *Escherichia* and *Streptomyces* (Chun *et al.*, 1993; Sisson *et al.*, 1995). ANNs are also considered to be relatively robust to noisy data, which would make the method useful for quantitative analysis (Goodacre *et al.*, 1996).

An example of multivariate data analysis from the authors' laboratory is presented in *Figure 1.4*. This figure shows the PCA score plot of PY-MS analyses from several different samples of agarose and four agarose-based liquid chromatography media. As can be seen in the figure, the first two principal components explain 94% of the variance, although 168 fragments have been used in the calculations. The four media are a series of samples taken during the production of the ion exchange media SP Sepharose™ 6 Fast Flow from agarose. *Table 1.3* shows the various stages in the production when the samples are taken. As can be seen in the PCA 'score plot', all media are differentiated from each other by two principal components. Sepharose 6B is very close to agarose, which is logical since the only difference between these two groups is that Sepharose 6B is in a bead-form whereas agarose is not. The data were

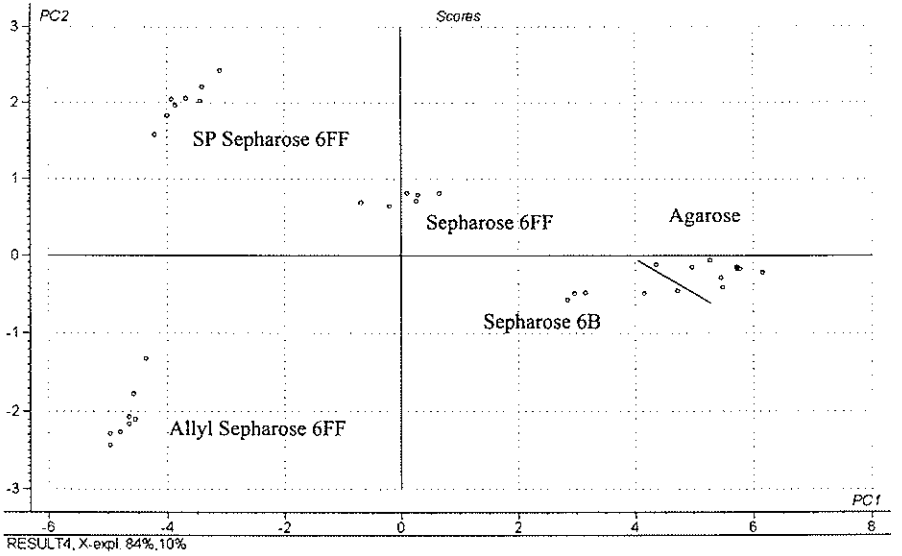

Figure 1.4. PCA scores plot from PY-MS analysis of four liquid chromatography media and agarose.

transformed using the standard normal variate transformation, SNV, method before principle component analysis. The SNV transformation method is usually applied to spectroscopic data to remove variations due to baseline fluctuations (Barnes *et al.*, 1989). In this case, by using SNV transformation, the sample differences due to variation in sample amount are better compensated for than when compared to using normalization of a specific peak or total peak area.

Amino acids, proteins and proteinaceous material

INTRODUCTION

There is a continuing need within biochemistry, medicine and biotechnology for the rapid identification and quantification of proteins (Aebersold, 1993; Goodacre *et al.*, 1994a). Despite this need, when compared to the numerous publications concerning pyrolysis in other fields, there seems to be a lack of interest in the analysis of proteins by pyrolysis. Conventional methods such as Kjeldahl titration, gas and liquid chromatography sometimes coupled to mass spectrometry, and other spectrometric methods,

Table 1.3. Description of differences between the four liquid chromatographic media analysed by Py-MS and PCA

Media	Description of differences between the media
Agarose	–
Sepharose 6B	Porous agarose beads
Sepharose 6 Fast Flow	Sepharose 6B + cross-linking using epichlorohydrin
Allyl Sepharose 6 Fast Flow	Sepharose 6 Fast Flow + linking of allyl groups
SP Sepharose 6 Fast Flow	Allyl Sepharose 6 Fast Flow + linking of SO_3^{2-} groups to the allyl

including fast atom bombardment spectrometry (FAB-MS), seem to have been preferred although they are tedious to perform compared to pyrolysis and require larger sample amounts (Chiavari and Galletti, 1992). Two possible reasons for this lack of interest have been suggested (Tsuge and Matsubara, 1985). Firstly, separation of the pyrolysis products, of which several are polar, has been considered to be 'difficult'. Secondly, investigators had found that the pyrolysis products of amino acids and related compounds were very sensitive to changes in the experimental conditions (Tsuge and Matsubara, 1985). However, solutions to the first problem have come with the advent of the inactive and highly efficient fused-silica columns for GC separation; and the second problem can be explained to a large extent in terms of the tendency of proteins to be completely denatured at temperatures around 200°C. As mentioned earlier, this temperature is often held in the pyrolysis chambers of flash-type pyrolysers to prevent condensation. By using a vertical microfurnace-type pyrolyser where the sample was not exposed to heat before pyrolysis, denaturing has been avoided and the reproducibility of the pyrograms has been improved (Tsuge and Matsubara, 1985).

Quite recently, the use of on-line tetramethylammonium hydroxide (TMAH) thermochemolysis (Zang *et al.*, in press) offered a significant step forward for the characterization of proteins and amino acids. Amino acids were converted to their corresponding *N*- and *O*-methyl derivatives by adding TMAH to the sample in the pyrolysis chamber and leaving it for 15 seconds at 250°C prior to separation and detection by GC-MS. A considerable benefit of this treatment is the volatility of the amino acid esters that are formed. This volatility makes it possible to analyse them directly by GC. Further heating (pyrolysis) is not necessary. It was also found that peptide bonds were effectively cleaved by the thermochemolysis reaction, yielding individual amino acid methyl esters. Thus, the method was also suitable for peptides, proteins and natural organic matter.

TMAH treatment in combination with pyrolysis has been frequently used in carbohydrate analysis for the past decade (Fabbri and Helleur, 1999), but has been very sparingly used for amino acids and proteins (Hendricker and Voorhees, 1998).

THERMAL DECOMPOSITION MECHANISMS

Amino acids

Since amino acids serve as the basic structural units for a large variety of peptides, proteins and enzymes, the pyrolysis of amino acids was of early interest (Úlehla, 1960; Winter and Albro, 1964; Kanomata and Mashiko, 1966). In those early studies it was demonstrated that analytical pyrolysis could produce unique pyrograms of amino acids and reveal detailed structural information.

The main mechanisms of thermal decomposition of amino acids are decarboxylation by CO_2 and water elimination with the formation of a dipeptide and further decomposition to diketopiperazines (DKPs). Several authors (see, for example, Moldoveanu, 1998; Chiavari and Galletti, 1992) have published lists of the main pyrolysis products of several amino acids. Most amino acids present in proteins produce compounds of diagnostic value, i.e. compounds produced only from the specific amino acid. However, low molecular weight amino acids such as glycine, serine, alanine and threonine yield rapidly eluting volatile fragments that are difficult to analyse by GC.

It is also possible that mutagenic compounds (heterocyclic amines) are formed during pyrolysis (Sugimura *et al.*, 1977; Basiuk and Navarro-Gonzales, 1997). These types of compounds have been detected in traces in the pyrolysates of amino acids, and the finding is of interest since amino acids, as components of proteins, are present in food.

Peptides

Pyrolysis of simple peptides can offer models for understanding the pyrolysis of larger polypeptides and proteins. Small molecules are obtained, not only similar to those formed from amino acids, but also DKPs and their secondary fragmentation products. The formation of DKPs takes place with neighbouring amino acids. The type and amount found in the pyrolysate is influenced by the nature and the stability of a particular amino acid in the sequence and by the pyrolysis temperature. Thus, by studying types and amounts of DKPs, structural information can be obtained. As can be expected, milder conditions favour the formation of larger fragments containing more structural information.

Proteins

Simple proteins form pyrolysis products similar to those generated from peptides. However, conjugated proteins demand extra attention: pyrolysis of such proteins does not always provide the desired information on the prosthetic group. For example, according to several authors the heme group is not revealed in the mass spectrum of hemoglobin (see Snyder *et al.*, 1988b). This is said to be due to its low contribution to the total mass (about 1%). The presence of carbohydrates in glycoproteins is not always obvious (Meuzelaar *et al.*, 1982; Munson and Fetterolf, 1987), at least not in Py-MS results, where fragments normally interpreted as resulting from the sugar might also originate from other sources such as acetates attached to the amino groups in the lysine or arginine residue.

Proteinaceous material

There is also a large group of proteinaceous-like material, such as from food (Lipp and Anklam, 1998), soil, (Schulten, 1996; Leinweber and Schulten, 1999), humic substances and paint sources (Chiavari *et al.*, 1993; Chiavari and Mazzeo,1999), that has been subjected to analytical pyrolysis. Problems related to the discrimination between different compounds in such materials are similar to those with conjugated proteins. In this field it has been especially important to find specific markers to be able to detect the presence or absence of the specific protein. Several researchers have contributed to the knowledge of protein markers (Boon and Leeuw, 1987; van Arendonk *et al.*, 1997; Pulchan *et al.*, 1997). The ions with m/z 154 and 168 are often used and have been proposed to be diketopiperazine compounds derived from dipeptides structures (Munson and Fetterolf, 1987).

THE USE OF DIAGNOSTIC MARKERS FOR DISCRIMINATION BETWEEN COMPOUNDS

The use of diagnostic markers, compounds or ions, is evidently of great help to interpret a pyrolysis result. However, there are several problems related to the

specificity or origin of diagnostic markers. For complex material, such as micro-organisms, two steps are required. A compound specific for the strain has to be found as a 'biomarker'. Then a specific pyrolytic fragment of that compound has to be found, as a 'pyrolytic marker'. Some examples of how to solve problems related to the markers are now considered.

Pyrolysis of paint layers is sometimes accomplished to characterize binding media. The use of diagnostic markers to recognize the binders is quite straightforward for binders belonging to different chemical groups. However, the diagnostic markers are not specific enough for discrimination between chemically similar binding media. Chiavari *et al.* (1998) proposed a semi-quantitative Py-GC/MS method to characterize such binders, using selective pyrolytic products representative of proteins, carbohydrates and lipids.

One way to solve, at least partially, the problem of determination of the origin of a specific ion fragment in Py-MS is to use temperature resolved data acquisition which separates mass peak distributions as a function of the thermal stability of the constituents in a sample. Van Arendonk *et al.* (1996) utilized this technique in a study of enzymatic removal of protein from plant leaf material. A platinum–rhodium filament in-source probe, directly inserted into the mass spectrometer, was used. The source temperature was 180°C at the start with a heating rate of 16°C/s up to a final temperature of 800°C.

Tandem mass spectrometry or time-of-flight mass spectrometry can be valuable to determine if a fragment ion is formed from more than one compound in the sample (Eglinton *et al.*, 1991).

CHARACTERIZATION AND CLASSIFICATION

Characterization and classification can be done by two techniques: comparing results from different samples or identifying the compounds in the pyrolysate. An example of the result of such a comparison has been given by Tan *et al.* (1995). Using Py-GC of washed and freeze-dried mononuclear cells of bone marrow from different patients, the authors succeeded in providing a simple, reproducible and rapid diagnostic tool for acute leukaemia. It was sufficient to study variations of some of the GC peaks of the pyrolysates to achieve this result.

Viral proteins have been differentiated by applying pattern recognition methods to Py-MS spectra (Tas *et al.*, 1989). Ogunsola *et al.* (1995) reported a correlation between Py-MS spectrometry results and outer membrane protein profiles of *Clostridium difficile*. They used discriminant analysis to type 50 different strains, and the typing successfully grouped >75% of the strains.

The articles below are examples of the second technique, compound identification. Py-GCMS was applied in a study of the composition, source and chemical changes of sinking particles in the ocean (Ishiwatari *et al.*, 1995). The GC peaks were identified by MS and, depending on the type of substance to which they belonged, they were classified into three classes of precursor: proteins (nitrogenous compounds such as pyrroles, pyridines, phenylalkyl nitriles and indoles), carbohydrates (furan derivatives) and lipids (alkenes, alkanes, nitriles, fatty acids).

The nature of the refractory organic nitrogen in humic acid from an organic-rich sediment was investigated for any proteinaceous materials encapsulated within the

humic acid structure by solid state NMR spectroscopy, Py-GC/MS, and TMAH thermochemolysis GC/MS (Zang *et al.*, 2000). The NMR spectra indicated a predominately proteinaceous nature and the two other techniques provided complementary evidence. In the chromatogram from the pyrolysis experiment the protein marker fragments with m/z 154 and 168 were found. The mass chromatogram of the TMAH thermochemolysis showed a series of amino acid methyl esters. Interestingly, the amino acids detected were mostly aliphatic, exactly those amino acids that are most difficult to study using ordinary Py-GC because of the low boiling point of their pyrolytic products. This demonstrates once again the usefulness of TMAH in protein analysis.

Quantitative pyrolysis

Much of the information obtained from the pyrolysis of proteins is related to the amino acid content and it has been shown that most of the amino acids produce diagnostic fragments that can be used not only for characterization, but also for quantification. For example, after pyrolysis of a protein sample, indole can be used for the determination of tryptophan, benzyl cyanide can be used for phenylalanine and the sum of phenol and *p*-cresol can be used for tyrosine (Marmer *et al.*, 1987). In the 70's and 80's the pyrolysates were mostly separated on a GC column before analysis. The number of Py-MS methods that have the advantage of being even faster is now increasing. An overview of quantitative analysis of Py-MS spectra using multivariate calibration and artificial neural networks was given by Goodacre *et al.* (1994b).

Reasons for using pyrolysis

As with pyrolysis in general, the speed of analysis and the feature of very little sample preparation required are very attractive. Some authors also appreciate the small amount of sample needed (Tsuge and Matsubara, 1985). The fact that pyrolysis is a multicomponent analytical method is sometimes important (Snyder, 1996; van Arendonk, 1997). Others have tried pyrolysis because other methods were not possible to use. The amino acid tryptophan is difficult to analyse using conventional amino acid analysis due to its sensitivity to acid hydrolysis. In several articles Marmer and coworkers (see Marmer *et al.*, 1987, 1989; Marmer and Magidman, 1990) have shown how the tryptophan depletion in wool processing could be followed by pyrolysis. Later, Schmidt *et al.* (1999) reported the successful analysis of milk samples with various whey protein contents using Py-MS. For these types of processed foodstuffs, other analytical techniques either gave uncertain results or were not possible to use due to heat denaturation, fermentation or matrix effects. However, the results from the pyrolysis method were reported to correlate well with the added protein content and to be uninfluenced by parameters such as fat content, freezing of the samples and dilution.

Reproducibility of quantitative methods

The reproducibility of quantitative methods is often expressed as the coefficient of variation (CV) or relative standard deviation (RSD). In an example, the repro-

ducibility of six lyophilized salt-free powders of enzymes, pyrolysed (resistively heated) at 800°C and analysed by GC-FID have been reported with a RSD of 2–10%. Figures of 2–10% are quite common for these types of methods. The enzymes were analysed in triplicate and the sample amount varied between 0.5 and 1 mg. The RSD was calculated using the five major peaks in the pyrogram normalized to the total height of the five peaks (Danielson *et al.*, 1978).

Some authors discuss problems with the reproducibility of quantification. Tsuge and Matsubara (1985) had found a correlation of variation (CVs) of 4.6 and 7.1%, respectively, for the tyrosine and tryptophan content in a protein. Those figures were mostly attributed to variations in the absolute masses (300 μg samples) since the CVs of the relative peak areas between the key components were found to be only 1–2%. Finding internal standards is often a problem. Internal standards need to be mixed with the sample and they have to be proven not to change the pyrolysis mechanism or produce the same fragments as the sample. Some suitable internal standards have been found. For example, benzothiophenone has been added to protein samples (Ohsawa *et al.*, 1988), and in the estimation of tryptophan in wool the inherent peaks from tyrosine were used as an internal standard (Marmer *et al.*, 1987). In Py-MS methods a normalization of the spectra to the total ion count can be done.

The precision can differ between compounds in the same method. In a method for determining sulphur-containing amino acids residues by Py-GC (Ohsawa *et al.*, 1988), the relative standard deviation for methionine was 5–6% while it was 10–12% for cysteine. This was said to be due to differences in thermal stability between methionine and cysteine residues in protein. Marmer *et al.* (1989) reported a CV of less than 3% for tyrosine, but 11% for tryptophan. In this case the reason for the discrepancy was believed to have been difficulties in integrating the small and unsymmetrical peak area for tryptophan. Reproducibility of the MS intensities of 10% was blamed on instrumental drift during the course of the day and pollution of the Pt/Ph wire was reported by van Arendonk (1997). The examples above all deal with difficulties. However, to put the situation into perspective, there are also cases in which the present authors have been satisfied. In an example from the food industry a method for the identification of soy protein in meat was developed (Raghavan *et al.*, 1986). Several peaks unique for the soy protein were found. Replicate analysis generated reproducible data with variations generally below the 5% level, which was considered to be very good.

Inter-laboratory reproducibility

When it comes to inter-laboratory reproducibility, an important area of concern is to cross-check results. This can be done by complementary analytical techniques, fundamental mechanical and/or kinetic studies of the thermal degradation of well-characterized samples, and standardization and compilation of a standard database for various series of standard samples (Tsuge, 1995). T.A. Gough and C.E.R. Jones showed in 1975, when analysing fragments of aged paint using different types of pyrolysers coupled to GC, that good inter-laboratory reproducibility could be achieved by strict observation of predetermined factors. E.J Levy and J.Q. Walker reported in 1984 that the copolymer Kraton 1107 could be used as a model molecular thermometer to compare and calibrate the true final pyrolysis temperature between different types of pyrolysers.

DNA

Much of what has been written about the pyrolysis of proteins is also valid for DNA. The chemical composition of DNA, of course, has an impact on the experimental conditions that should be chosen, but, just as for proteins, one of the benefits of using pyrolysis compared to other methods is the rapid procedure. Past studies of pyrolysis on DNA have focused on the identification and detection of minor nucleotides, sequence analysis, discrimination between closely similar oligonucleotides, structural characterization and quantification. Several of the reports on discrimination of closely similar oligonucleotides (Freeman *et al.*, 1994) have been performed on whole cells and are considered in the section below on 'microorganisms'.

Nucleotides consist of a carbohydrate linked via a β-D-glycosidic bond to a heterocyclic base and to a phosphate group at C-5. Nucleotides are highly polar, which decreases their volatility and presents a challenge to mass spectroscopists. A dominance towards either the base or the sugar moiety can be achieved, depending on the pyrolytic conditions. Experimental conditions have been studied by Snyder *et al.* (1988a).

In the seventies and eighties DNA-containing samples were often directly introduced into the mass spectrometer. Temperatures of between 200 and 300°C, which are normal in the ion source, were enough to obtain some fractionation, or at least partial vaporization of the sample (Jarman, 1980). Cleavage is reported to proceed in two steps (Macquet *et al.*, 1980). First the phosphodiester bonds are cleaved and then there is a cleavage of the nucleoside, giving products that can be used as diagnostic ions for base fragments. This method was quite successful and there are reports on classification of platinum–DNA complexes (Macquet *et al.*, 1985) and monitoring of synthesis of DNA fragments using this technique.

The direct inlet technique described above contains mainly ions characteristic for the bases. However, it has been shown that ions characteristic for the sugar moiety (Meuzelaar *et al.*, 1982) are given by Curie-point Py-MS. On the other hand, when using this technique there are few ions characteristic of the base. It is thought that there is a formation of base–phosphate conjugates during the pyrolysis which condense on the walls of the reaction tube (Snyder, 1988a). Heating the reaction tube above 500°C results in the formation of free bases and non-volatile (poly)phosphates.

The development of mass spectroscopic techniques such as electrospray-mass spectrometry (ESI-MS) and matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) in recent years has directed analysis of nucleosides and nucleotides to those techniques and interest in pyrolysis has decreased. However, in 1996 a paper was published which might again draw attention to pyrolysis (Abbas-Hawks and Voorhees, 1996). In that study, nitrogen bases in free nucleotides, oligonucleotides, calf thymus DNA and whole bacterial cells were reacted *in situ* during pyrolysis at 510°C with TMAH to form the methylated bases. There were several advantages, the most important of which was that the volatility of the nitrogen bases increased. The mass of the diagnostic base peaks also increased, which removed them from the positions of lower-mass background peaks. This method could therefore be used successfully to identify DNA components in a broad spectrum of samples.

Quantitative results on DNA can be obtained by pyrolysis (Goodacre and Kell,

1996). It has been shown that nucleic acids, primarily DNA, are the main source of furfuryl alcohol in pyrograms of bacteria (Eudy, 1985). In 1992 a Py-GC/MS method for determination of DNA in cultured mammalian cells, based on measurement of furfuryl alcohol, was published (Sahota *et al.*, 1992). In this method an internal standard, *N*-acetylglucosamine, was used to account for variations in sample size. The internal standard was pyrolysed along with the sample. The detection limit was reported to be about 100 ng DNA. The results of the Py-MS method were also compared with those from a conventional, colorimetric diphenylamine method. The Py-GCMS method gave higher values, but otherwise correlated satisfactorily with the colorimetric method. However, the reproducibility was poor for both methods. This could be due to inhomogeneities in the cell lysates and not to do with the quantification methods themselves.

Characterization of microorganisms

Conventional methods for characterizing and identifying microorganisms normally involve incubation of microorganisms to obtain a pure culture, followed by a wide variety of time-consuming morphological, serological, nutritional and biochemical tests. Methods to characterize a large number of microorganisms quickly and accurately are therefore required. A promising approach is chemotaxonomy. In chemotaxonomy, chemical and physical techniques are employed to produce a chemical profile of taxonomic significance. Analytical pyrolysis was proposed as early as 1952 (Zemany, 1952). However, the development of low-cost, fully automatic pyrolysis mass spectrometry (Aries *et al.*, 1986) and improved data handling facilities enabled the technique to be used as a tool for the classification, identification and typing of microorganisms (Goodfellow *et al.*, 1994; Magee, 1994; Goodacre and Kell, 1996). Viable organisms are not required, there is minimal sample pre-treatment and short total analysis time.

SAMPLE PRE-TREATMENT AND PYROLYSIS

There is one type of pre-treatment that seems to be used increasingly in this area as well as in other fields of analytical pyrolysis. This is the use of tetramethylammonium hydroxide (TMAH). In the case of chemotaxonomic analysis based on the fatty acid distribution, the fatty acids are derivatized to their methyl esters before analysis. To avoid sample extraction at derivatization steps and to speed up the analysis time, TMAH can be added to the microorganisms prior to pyrolysis (Basile *et al.*, 1998). This procedure means that the fatty acids are methylated (*in situ* thermal hydrolysis methylation-THM) during pyrolysis of the microorganisms (Beverly *et al.*, 1997).

In addition to different sample treatment procedures, many forms of pyrolysis techniques can be used and it is important to understand their drawbacks and merits.

ANALYTICAL TECHNIQUES FOR ANALYSING THE PYROLYSATE

The characterization of bacteria with pyrolysis techniques has largely been approached with Py-GC, Py-MS and Py-GCMS. The simplest and most rapid of these methods is direct Py-MS with electron ionization. A drawback is that the Py-MS spectra are

complex and do not lend themselves to easy chemical interpretation. Py-GCMS allows identification of specific biomarkers important for the discrimination of microorganisms, but is not as fast as Py-MS. Tandem mass spectrometry can, to some extent, combine the advantages of the two techniques (Voorhees *et al.*, 1992) by permitting identification in a pyrolysate, while at the same time retaining the speed of traditional Py-MS.

Much of the previous work with MS detection has been done using electron ionization of the pyrolysate. Chemical ionization for differentiating microorganisms has not been applied so often. Electron ionization causes extensive fragmentation, resulting in a high abundance of low mass ions and diminishing the diagnostic value. In chemical ionization the fragmentation due to ionization is less. This enhances the molecular ion region for fatty acids (Barshick *et al.*, 1999).

An interesting detector for Py-GC is the ion mobility spectrometry system (IMS). Although Py-GC/IMS cannot compete with mass spectrometry based pyrolysis techniques in terms of specificity, the difference in size, power requirements and cost can make up for these shortcomings under many possible application scenarios (Snyder *et al.*, 1996). A field-portable Py-GC/IMS has been used to provide real time detection of Gram-positive bacterial spores used as biological threat agents, for example *Bacillus anthracis*.

STRATEGIES FOR CLASSIFICATION

Data handling of the pyrolytic results from microorganism samples is a very important element in taxonomic studies. Techniques described earlier in this article can be utilized. The classification will be simplified by identifying specific compounds in pyrograms as originating from relevant microbial structures. If such specific compounds can be found, variations due to growth conditions, treatment, sampling conditions and instruments will be reduced (Voorhees and DeLuca, 1991). The kind of chemical markers that can be utilized in analytical pyrolysis include carbohydrates, DNA, proteins and lipids. The microbial polar lipids are particularly useful for taxonomic purposes. On the other hand, proteins and nucleic acids, although sometimes typical for a certain species, do not have specific amino acid or nucleotide content to make them optimal to differentiate microorganisms. The chemical composition of the cell wall, outer membranes, and capsules of bacteria is sufficiently diverse with respect to the markers mentioned so as not to disturb discrimination between different microorganisms.

Much research has been done in the past ten years to study the unique pyrolysis signature from specific microorganisms (Morgan *et al.*, 1995). A long-term goal is to produce pyrolysis data on markers present in a wide variety of organisms and to construct a library to enable automated pattern matching.

VALIDATION OF TECHNIQUES

Although many microbial pyrolysates have been identified, few markers have been validated in a proper way. For example, the importance of testing multiple strains from each group of microorganisms to check reproducibility of results within the group has not been adequately addressed. Furthermore, the relevant pyrolytic product

Table 1.4. Summary of some pyrolysis products and biomarkers from different microorganisms

Microorganism	Pyrolytic marker	Biomarker	Reference
B. cereus, B. circulans, B. licheniformis, B. sphaericus, B. subtilis, B. thuringiensis, S. aureus, S. epidermidis, S. pyogenes, P. aeruginosa, P. cepacia, P. putrefaciens, P. stutzeri, E. coli, etc.	Fatty acids: C(14–19):0, iso C(14–19):0, C16:1, anteiso-C17:0, methyl esters of fatty acids (C14–18):0	Cell membrane	DeLuca *et al.*, 1990; Voorhees *et al.*, 1997
Bacterial spores	2-Pyridinecarboxylic acid	2,6-Pyridine-dicarboxylic acid	Thornton *et al.*, 1994; Snyder *et al.*, 1996
B. subtilis, M. luteus, S. longisporoflavus	Acetamide, propionamide	Peptidoglycan	Simmonds, 1970; Medley *et al.*, 1975
L. pneumophila, A. hydrophilia, P. aeruginosa, B. anthracis, B. globigii, B. subtilis, S. aureus, etc.	Furfural alcohol	DNA, RNA, carbohydrates	Morgan *et al.*, 1990
S. agalactiae (Group A and B)	Di-anhydroglucitol	Group-specific polysaccharide	Morgan *et al.*, 1990
B. globigii, B. subtilis, E. coli	Adenine	Nucleic acids	Voorhees *et al.*, 1992
B. globigii, B. subtilis, E. coli	Diketopiperazine, indole	Proteins	Voorhees *et al.*, 1992
B. anthracis	1,6-anhydro-galacto-pyranose	Galactose	Morgan *et al.*, 1995
L. pneumophila, P. vulgaris, B. anthracis	2-Butenoic acid	Poly-β-hydroxy-butyrate	Watt *et al.*, 1991
B. calopus and *B. bovinus* fungal spores	Toluene, hexadecanoic acid, saturated and unsaturated hydrocarbons	Lipids	Papa *et al.*, 1989

must be related to the biomarker to know the chemotaxonomic features. It is therefore essential that pyrolysis data of whole bacterial cells be compared with data from known biomarkers and appropriate cell fractions (Morgan *et al.*, 1990). In *Table 1.4* some identified pyrolytic products for different microorganisms are presented.

FUTURE DEMANDS

In conclusion, the application of Py-MS to microbiology is undoubtedly useful to discriminate between bacteria at the genus, species and subspecies level. However, it is still a long way before pyrolysis can become a general identification technique for microorganisms. One reason for this is that microorganisms of the same species exhibit large natural variability in chemical composition. For a pyrolytic product to be an optimal marker for discrimination, it must be an invariant feature. This means, for example, that distinctive pyrolytic products could be generated under a wide range of experimental conditions. It is therefore of utmost importance that standard reference materials of microorganisms, cell fractions and model compounds are available to establish the pyrolysis method and a model for the evaluation of pyrolysis data. Detailed analyses of pyrolysates are needed to characterize the cell constituents that contribute to the spectra and hence to the differentiation of organisms.

Concluding remarks

This review may appear to have focused on the problems and difficulties associated with pyrolysis. However, the intention is not to advise against using the technique. Instead, it is believed that by knowing what problems to expect, many of them can be easily avoided. It will also be easier to decide when pyrolysis is worth considering: it has long had the advantages of being a fast, sensitive, easily automated, multi-component technique. In recent years, the use of TMAH together with the development of low-cost, fully automatic pyrolysis mass spectrometry and improved data handling facilities has increased the range of applicability of analytical pyrolysis. The potential of these improvements has not yet been fully exploited.

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