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Mass Spectrometry for On-line Monitoring of Biotechnological Processes

E. Heinzle*

Institute for Environmental Research, Elisabethstr. 11, A-8010 Graz, Austria

* New adress: Chemical Engineering Laboratory, ETH-Zentrum, CH-8092 Zürich, Switzerland.

1 Introduction

1.1 On-line Data Acquisition and Control: Measurement Technique Problems

Biotechnological process investigation and operation is usually very expensive. Most of the processes are rather slow, often lasting a couple of days and aseptic operation is absolutely necessary to avoid competition from undesirable organisms. The media used may be very expensive, especially so in the case of animal-cell cultures which often require large amounts of fetal calf serum. For these and other reasons it is very desirable to get as much information as possible out of each experiment and to closely monitor or even control production processes in order to minimize costs and product quality. Product quality may be improved by increased selectivity, increased product concentration and increased reproducibility which may significantly reduce costs for down-stream processing.

Information may be collected by batch-sampling and subsequent chemical and/or physical analysis. This involves a number of manual operations but is most flexible and therefore the primary step to process analysis. For continuous collection of information this method is obviously not suitable and its applicability to control is limited to very slow processes and crude control methods. Automatic sampling can especially simplify over-night operation, but real improvement in process monitoring and control may be obtained by the introduction of automatic analysis. Automatic sampling increases risks of infection. Separation of liquid from cellular material and other solids still creates a lot of problems though much progress has been made recently [33, 66, 74, 103]. For process control, on an industrial scale especially, the system described by Lenz et al. [66] seems to solve most of the problems. It involves filtration analysis for biomass estimation and on-line use of filtered samples for direct high performance liquid chromatography (HPLC) analysis.

Obviously the most desirable method for process monitoring is the application of continuously operating sensors. These should have a number of characteristics:
— The specificity should be high enough to give a useful signal for the concentration of one single chemical species or a group of them.
— Because of low concentrations, especially at the initial state of projects, the sensitivity must often be very high.
— In most cases steam sterilizability will be a necessary precondition.
— The sensor should not interfere with the process.
— Accuracy requirements will often be moderate (1–10 % relative).
— Long term stability will reduce the need for recalibration, which under sterile conditions would in any case be very cumbersome.
— Sensor costs and maintenance requirements may be practical limits for certain applications.
— Dynamic characteristics will play an important role if the measured variable changes rather quickly. As a rule of thumb a sensor whose time constant is about 4–5 times larger than the time constant of the process itself can be used without significant problems. If the sensor has a first order delay characteristic the dynamic relative error would be $<2\%$.

To control a process the sensor will be an integral part of the feed-back control loop.

The quality of the sensor must be related to the process characteristics and the requirements for control performance. If the process is a completely black box, i.e. if no descriptive model exists, the variable to be controlled to desirable values must be measured directly. If some characteristics of the process are known, a model can be built which eventually allows the measurement and control of a related variable to finally control the desired variable. Estimators have been applied to allow calculation of interesting process variables [78,112,127,157]. Unfortunately in biotechnical processes the knowledge of the process mechanism is often very limited.

On-line estimation of variables may often be improved considerably by applying filter techniques [29,30,42,100,114].

1.2 Present State of Measurement Technique and Trends

A number of automatic analytical methods are now available on the laboratory scale as can be seen from a few examples listed in Table 1.

The performance of most of these methods is strongly dependent on sampling quality. In general all methods [6,33,63,66,74,82,119] are suitable for liquid separation, but dialysis membranes may be very fragile and some other filter probes may be subject to membrane fouling during long term operation. One method uses fresh filters for each sample [6,66] in order to avoid fouling. This probe, however, requires a large sample volume and cannot be easily used on a laboratory scale. Detecting methods may greatly vary (HPLC [6,33,66], autoanalyzer [119], IR [1], enzyme thermistor [28], MS [91] and others).

Conventional automatic analyzers would often need too large amounts of samples, which could be somewhat reduced by moving from segmented flow to flow injection

Table 1. Examples of automatic analysis in biotechnological processes

Variable	Method	Ref.
Biomass	Filtration probe	82)
Biomass, dissolved substrates and metabolites	Filtration probe and HPLC	6) 66)
Dissolved substrates and metabolites	Filtration cell and HPLC	33)
Ammonia, phosphate, glucose	Dialysis with enzymatic and chemical detection	119)
Penicillin, sugars	Enzyme thermistor	28)
Glucose	Dialysis with enzymatic detection	74)
Volatiles in the gas phase	Headspace/GC	23)
2-Oxoglutarate	Esterification/MS	89)
Glucose, ethanol, glycerol	FTIR/ATR	1)
Ethanol	Gas phase IR	80)
Extracellular enzymes	Ultrafiltration cell	63)

Table 2. Sensor for bioprocesses

Variable	Method	Ref.
Biomass; NAD(P)H	Fluorescence probe	5, 142)
Ethanol	Enzyme probe	121)
Glucose	Autoclavable enzyme electrode	21)
Penicillin, glucose	Enzyme electrode	34)
Citric acid production	Redox-electrode	4, 62)
Methanol	Silicon tubing	141)
Volatiles and dissolved gases	Porous Teflon tubing	27, 43, 139, 140)
Dissolved CO_2	Electrode	88)
Dissolved O_2	Electrode	52)

analysis. This also usually leads to a speeding up of analysis. Recent progress in miniaturization of biosensors leads us to expect significant progress in the field of continuous monitoring in a sampling stream, and may not require performance under aseptic conditions. The prospects in this field include miniaturized multicomponent probes which are not sterilizable and therefore very inexpensive and disposable. If monitoring of volatiles is of interest, detection in the gas phase may be much easier [23, 80], but liquid-gas phase dynamics may cause errors in this case.

There are already a number of sensors that are used in bioprocess monitoring. These, however, are mostly sensors for physical variables and some of them are not directly steam sterilizable (Table 2). There exists a fairly long list of other sensors which are not sterilizable or subject to cross interferences (e.g. biosensors, ion-specific electrodes). Well established methods such as measurement of temperature, pressure, and pH are not listed here.

It can easily be seen that only a few sensors can directly measure product or substrate concentration under sterile conditions. Biomass concentration or activity cannot usually be measured on-line.

Present trends towards the development of new methods or for improving existing ones are classified in Table 3.

Table 3. Trends in on-line measurement

— Improvement in sampling techniques to couple powerful analytical methods to processes (HPLC [66], enzymatic sensors, automatic chemical analysis).
— Application of chip production technology to develop new miniaturized sensors. These may be multicomponent sensors being not steam sterilizable but very cheap. They can be used in very small amounts of sample stream to continuously monitor a series of variables in the non sterile region of a process.
— Adaptation of existing analytical methods (fluorimetry, laser technology [113], mass spectrometry, infrared spectroscopy [1]).
— Application of balancing methods to calculate new variables from available sensor signals [97].
— Incorporation of suitable mathematical filter techniques may further improve calculated variable accuracy [29, 30, 42, 101, 114].

2 Mass Spectrometry Applications in Fields Other than Biotechnology

2.1 Analysis of Gases and Volatiles

Mass Spectrometry (MS) has a long history dating back to since the beginning of this century. According to Brunnee and Voshage [14] the first quantitative gas analysis of volatile hydrocarbons was carried out in 1940. In 1949 MS had already been successfully applied to breath analysis [54] and later in chemical process monitoring [19, 86, 106]. Breath analysis is still an important field of on-line MS application [81]. Speed of detection and the inherent ability of MS to simultaneously analyze several gaseous compounds are the main advantages in this case. The application of MS for analysis of steel processes to monitor the progress of oxidation of carbon is a very well established method [19, 106, 111]. This example shows that MS can be used in a harsh industrial environment. In chemical processes MS may be superior to gas chromatography and other methods when speed of analysis is crucial e.g. in the production of ethylene oxide, propylene oxide, acrylonitrile, vinyl acetate and vinylchloride [25, 26, 37, 83, 108, 109]. Multala et al. [79] applied MS analysis to computer control of distillation. Brockman and Anderson [13] used permeable membrane MS probes to monitor volatile products of electrochemical reactions.

In medical areas other than breath analysis, a great deal of effort has been applied to blood gas monitoring using membrane probes [11, 64, 126, 137]. Monitoring of blood gases and volatile anaesthetics may be very attractive during critical operations. More recently non-invasive transcutaneous measurement of oxygen and helium dissolved in arterial blood using a specially designed membrane probe was described [40]. Weaver [130] gave a review on the medical and other applications of MS for measurement of volatiles. Langer et al. [65] measured dissolved gases in plants.

MS also has been applied in the monitoring of atmospheric pollution [75, 84]. The greatest current use of MS for process controls is submarine atmosphere control [138].

2.2 Isotopic Studies

MS is ideally suited for monitoring isotopes. Radioactive isotopes however can usually be detected more easily and more sensitively by measuring radiation. Often it is advantageous to measure stable isotopes e.g. to avoid radiation hazards or for those elements which lack radioactive species. The most useful isotopes are ^{2}H, ^{13}C, ^{15}N, ^{17}O and ^{18}O [90, 93, 110].

2.3 Complex Mixtures

MS has also been applied in the direct analysis of complex mixtures though it is inherently limited in this respect because of overlapping of peak fragments from individual components. Despite this limitation, MS was successfully used to continuously analyse natural gas samples [123]. A general method was developed to improve analysis

of mixtures [105]. MS was coupled to pyrolysis ovens to analyze coal [120], to classify microorganisms [2, 135], to analyze polymers and polymer mixtures [136] and to analyze other complex biological samples [76].

3 Principles of MS Operation

The principles of MS operation are discussed in a number of text books [14, 61]. In this article only a few important aspects with relevance to on-line analysis in fermentation will be discussed briefly.

Figure 1 shows schematically the units necessary for MS use in process monitoring.
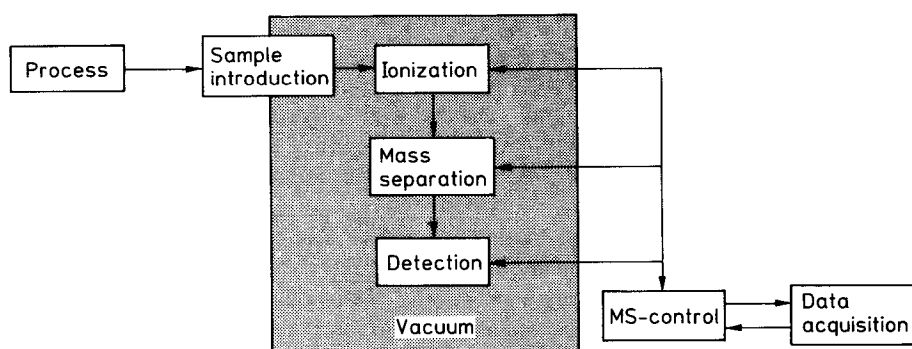
Fig. 1. Basic requirements for process MS

3.1 Vacuum

In MS, ions are used which will only survive sufficiently long if the vacuum is good enough. High pressure would allow collision with other molecules causing unpredictable reactions. The vacuum needed in the analyzer chamber will usually be $< 10^{-5}$ mbar. Below this pressure the mean free path of particles will be sufficiently long and the number of collisions sufficiently small to allow good analysis (e.g. N_2: mean free path ≈ 0.5 m and the number of collisions of one molecule ≈ 70 per s, in this case the time from ionization to detection is < 10 ms).

The required vacuum may be produced by several methods: three will be briefly discussed. The most traditional and simplest is a combination of a rotary pump to produce about 10^{-3} mbar and an oil-diffusion pump to evacuate to the required working pressure. A turbomolecular pump may be used instead of the diffusion pump. Turbomolecular pumps give a very low contamination by hydrocarbons and other compounds. Oil-diffusion pumps need cold baffles to avoid diffusion of oil into the analyzer and this either necessitates cooling with liquid nitrogen, methanol dry-ice or the application of Peltier-cooling. Accumulation of condensed material can cause long term problems. Turbo pumps on the other hand, involve high rotation speeds of

about 200 s^{-1}: their bearings will have a limited life-time, but prophylactic exchange of bearings after defined periods may avoid damage to the rotor and stator packages. Such damage would entail high costs. Start up to turbo-pumps is very fast and they can withstand rapid flooding with air. The pumping speed of the above pumps is mainly a function of the mass of molecules to be pumped. Sorption pumps do not rely on any movable parts except at start up. Their characteristics are also dependent on the chemical nature of the compounds to be pumped: noble gases in particular may be pumped at greatly reduced rates. The life-time of such pumps is limited by their sorption capacity and according to manifacturers will be about one year.

3.2 Ionization

A great number of ionization methods have been used [61] but for continuous operation and quantitative analysis electron impact ionization (EI) seems to be the most useful [108]. EI can give stable operation over a long period of time [45]. One important requirement for obtaining stable operation is constant temperature [61]. As most biological samples may contain oxygen, proper selection of cathode material will enhance the life-time. A gas-tight ion source with cathode filament outside the ionization chamber but within the high vacuum region ($< 10^{-6}$ mbar) will increase the life-time of the filament. Because of this most process instruments have closed ion sources. With proper installation and selection of filament material, especially with respect to corrosion by oxygen, filaments can be continuously operated for one year or even more. Practically any chemical compound can be ionized by EI provided the molecule can be introduced in the gas phase into the high vacuum. The energy applied to molecules is usually very high (70 eV) and therefore extensive fragmentation
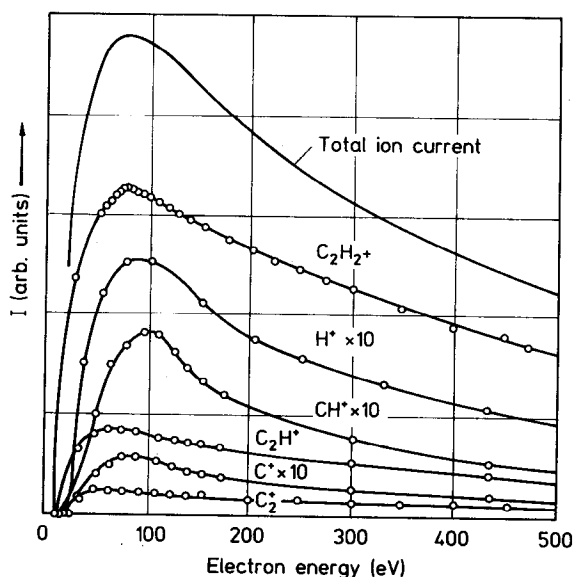

Fig. 2. EI ionization yield of acetylene as function of ionization energy [61]

Table 4. Ionization methods

Method	General applicability	Problems	Process use
Electron impact (EI)	Gas phase	Fragmentation, complex spectra	Extended and stable operation possible
Field ionization (FI)	Gas phase	Sensitive to dirt	Not suitable
Field desorption (FD)	Mainly solid samples	Only batch	Not suitable
Laser (LI)	Gas and solid phases		Generally possible
Photo (PI)	Gas phase		Generally possible
Thermal (TI)	Mainly solid samples		Not suitable
Thermospray (TS)	Liquids containing ions	Capillary sensitive to dirt and salts	Not suitable
Chemical (CI)	In the gas phase at higher pressure	Reactivity of the reagent gas depends on compound	Possible

is observed. Lowering the energy input favours higher masses which will be especially useful when analyzing mixtures. But fragmentation will still be considerable. In Fig. 2 ionization of acetylene is shown as a function of electron ionization energy.

It would be desirable to use soft ionization techniques which use much lower energies to ionize. These ideally would give only one single peak (e.g. molecular peak) from a single chemical species. A number of such soft ionization techniques are described in the literature and most of them also are commercially available. Recently it was claimed that chemical ionization has been developed to a state which allows process on-line monitoring [143]. Table 4 gives a list of ionization methods and a brief evaluation of their applicability for biotechnological process monitoring.

From Table 4 we can see that in the foreseeable future only EI, photo-ionization and chemical ionization will be of practical importance in this field. Chemical ionization is very attractive, but obviously water greatly disturbs the reproducibility of measurement [109].

3.3 Mass Separation

At present two methods dominate on-line application, magnetic sector and quadrupole mass separation. Both methods are technically well developed and in most cases equally suitable.

Quadrupole Instruments
The quadrupole consists of four rods ideally of parabolic shape. A voltage consisting of a DC and a radio frequency component is applied. Opposite rods are connected. Positive ions entering the oscillating field will move on oscillatory paths. Only ions with a particular mass will move on stable paths, others with higher or lower masses will be discharged on collision with the rods of the quadrupole. Mass selection is achieved simply by setting the DC voltage e.g. between 0–10 V. Quadrupole instruments can be made very compact and sensitive. These instruments are ideally suited for very fast selected ion monitoring (SIM) or multiple ion detection (MID).
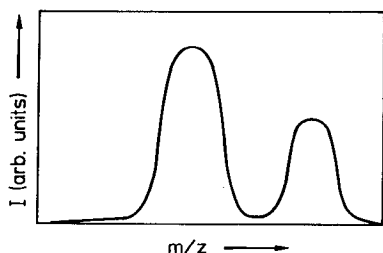
Fig. 3. Typical peak form of a quadrupole MS

In a quadrupole MS trapezoidal peaks cannot be obtained. The peak form can be manipulated to a certain degree by setting appropriate electric parameters. Figure 3 shows typical quadrupole peaks observed in practice. Using computer control of the MS slight disturbances in m/z (the mass to charge ratio) can be easily compensated for by occasional recalibration.

Quadrupole instruments are most frequently used in process gas analysis because they are cheaper and easier to use [109]. Quadrupoles of compact construction will allow operation at pressures up to 10^{-3} Torr [3] because a shorter mean free path of the molecules may be tolerated.

Magnetic Instruments

If a charged particle enters a magnetic field it will move on a circle whose radius is a function of the magnetic field, the particle velocity, and its mass to charge ratio (m/z). If the components to be measured are well defined and are not changing, fixed magnetic field and acceleration voltage combined with a number of simultaneously operating detectors may be used. More flexibility is obtained however, if scanning of either magnetic field strength or acceleration voltage is possible. Magnetic field scanning was improved by the introduction of Hall effect sensors. Both scanning methods are more complicated than changing the DC component of the quadrupole voltage. Magnetic instruments of appropriate construction can give higher resolution, and this resolution can be further improved by the addition of an electric field sector. Such a process instrument applied in trace gas analysis was recently introduced [138]. Generally magnetic instruments require more complicated ion sources because ions entering the magnetic field should have uniform energy. For quantitative analysis modern magnetic sector instruments are preferred [109] because the MS parameters can be selected to obtain trapezoidal peaks (Fig. 4).
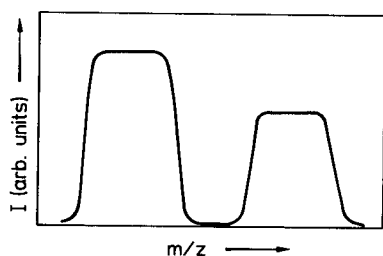

Fig. 4. Attainable trapezoidal peak form in magnetic sector instruments

This is possible if slits at the exit of the ion source and at the detector are properly selected. Because of the trapezoidal peak form, quantitative results are independent of small disturbances in m/z due to disturbances in acceleration voltage or magnetic field strength.

The greatest current use of MS for process control is for submarine atmosphere control [138]. These instruments were further developed recently by the addition of an electric sector to improve analysis of trace gases.

Other MS Types

Recently an ion trap detector especially designed for coupling to capillary-GC came on the market [36]. This very compact instrument may eventually be worth investigation for process applications because of its low susceptibility to changes in resolution by deposition of material on surfaces. Also, because of its compact construction, operation at slightly higher pressures is possible.

For future applications, Fourier transform (FT) MS may be very attractive. Smaller and cheaper instruments with their extremely good resolution may allow resolution of peaks which would usually overlap. Presently these instruments are too large and too expensive and sample introduction is critical.

Time of flight instruments seem to have certain advantages especially because of insusceptibillity to pollution but applications are very rare [25, 26].

General Aspects

Resolution may play an important role in quantitative MS operation especially if high accuracy is required or if small peaks in the neighbourhood of large peaks have to be measured: the tail of the large peak may considerably influence the intensity of the smaller one. Resolution (m/Δm) e.g. at 10 % valley is constant over the entire mass range in magnetic instruments. Quadrupole instruments are usually tuned to keep Δm constant. Δm will usually be 1, which means that neighbouring peaks of full masses are resolved over the whole mass range. This is obviously not possible if intensities differ very much. Quadrupoles usually give a certain mass discrimination at higher masses, which will be compensated during calibration.

Statistical errors of intensity measurement can be reduced by increased measurement time at the peak maximum. As the peak maximum is proportional to the peak area, (actually the basis for quantitative evaluations) integration of whole peaks is not necessary. But if measurement at the peak maximum cannot be guaranteed, whole peak integration can improve measurement accuracy in the absence of large neighbouring peaks.

3.4 Detection

Ions can be directly collected by a plate or Faraday cup to give an electric current which has to be amplified using an electrometer amplifier. Good long term stability, but relatively slow speed and limited sensitivity are the characteristics of such detection. Secondary electron multipliers (SEM) give additional very fast amplification of several orders of magnitude (10^4–10^8). This allows much faster operation and increased sensitivity of measurement. One major disadvantage of using an SEM

is the aging process which especially intitially may cause a significant decrease in sensitivity. In our experience after some time of operation the SEM seems to become more and more stable. Ideally, automatic switching between SEM and Faraday cup will be used: this has been achieved in modern instruments [138].

SEM or channeltron multipliers can also be used for ion counting which is applied for high speed scanning with relatively small ion signals. The counting method has a very high dynamic range and is much less sensitive to noise originating from vibration or to drift because of aging of the detector. High intensities ($> 10^7$ ions s^{-1}), however, cannot be measured with currently available equipment [76].

3.5 Data Handling

Multiple Ion Detection

Ion signals collected from ion current measurements can be treated in a number of different ways depending on the purpose. The simplest method is recording of spectra or single peak intensities using an oscillograph or an analogue recorder. With micro-electronics which usually will be used in modern instruments analogue signals will be digitized and can then be used in a number of ways. If selected ion monitoring is applied signals can be demultiplexed and a multichannel recorder can be connected to sample and hold circuits. Digital signals can also be used for computer treatment which may involve averaging, data storage and further mathematical treatment [20].

Analysis of Mixtures

If mixtures with superimposed ion fragment intensities have to be analyzed, a system of linear equations has to be solved to calculate concentrations of individual components in the mixture. The general method for quantitative analysis of mixtures with n components with concentrations $c_1, c_2, ..., c_n$ assumes linear superposition of peak intensities. The measured ion currents l_j for all masses are considered to be the sum of the contributions of all components, which are proportional to the concentration of each component, and to sensitivity coefficients $s_{j,1}, s_{j,2}, ..., s_{j,n}$:

$$l_1 = s_{1,1}c_1 + s_{1,2}c_2 + ... + s_{1,n}$$
$$l_2 = s_{2,1}c_1 + s_{2,2}c_2 + ... + s_{2,n}$$
$$.$$
$$.$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad (1)$$
$$.$$
$$l_m = s_{m,1}c_1 + s_{m,2}c_2 + ... + s_{m,n}$$

In matrix notation we can write

$$\mathbf{I = SC} \qquad\qquad\qquad (2)$$

In principle it is sufficient to measure $m = n$ suitable ion currents to determine C. The matrix S has to be determined by calibration with all the individual pure components. It can easily be seen that errors will be minimal if peaks are not overlapping, i.e. if only "pure masses" with a contribution from only one single component are measured.
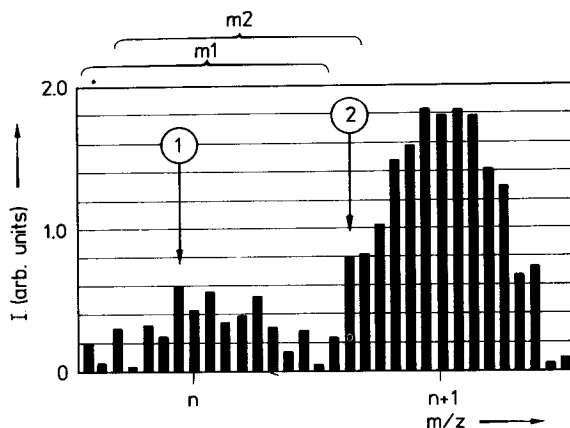
Fig. 5. Noisy measurement and detection of peak maxima. 1: ion current; m1, m2: masks for maximum selection; 1,2: maxima found using masks m1 and m2 respectively

If peaks are overlapping, several methods are described to reduce errors. Voogd et al. [123] first calibrated with pure components of natural gas getting absolute errors of 0–2 %. After additional calibration with a suitable gas mixture similar to natural gas they calculated a correction factor for each component. Following this procedure the absolute errors were reduced to 0–0.2 %. Schorr et al. [105] used statistical methods to calculate errors in analysis of gas mixtures and to minimize them. Breth et al. [12] showed that cracking patterns may be influenced by a number of parameters other than ionization method and energy.

Spectral Scanning

For scanning of whole spectra or parts of spectra, the maximum intensity for each mass unit has first to be selected from primary data already existing in digital form (e.g. 16, 32 or more measurements per unit mass). This can cause problems if instruments are not sufficiently well tuned or if the actual mass is in between two neighbouring integer masses. Especially in the lower intensity region and at high scanning speed, double peak detection can occur because of measurement noise (Fig. 5). If the mass is in between two neighbouring integer masses, the mask for maximum selection can be shifted. This can be especially critical if small peak maxima in the neighbourhood have to be found by automatic procedures. This is illustrated in Fig. 5, where a shift of the mask for maximum search gives different maximum selection with wrong m/z and intensity. In the case of measurement problems because of noise at low intensities, scan speed can be reduced or the threshold value for peak detection can be increased. This would lead to disappearance of smaller peaks thus reducing the information collected.

Sets of spectra can be analyzed using a number of methods. Library systems (which nowadays are also available for microcomputers) can be used to make attempts to identify single components or to compare spectra with those already stored in