Laser desorption analyses in trapped ion mass spectrometry systems
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5

Design and Performance of an Arbitrary Waveform Generator for Ion Excitation in Trapped Ion Mass Spectrometry

The design of a novel arbitrary waveform generator, which has been developed for the excitation of ion motions in ion traps, is presented. The system is equipped with a 192 Mb solid state memory for the storage of excitation waveforms. A local processor ensures its real-time operation and controls the waveform generation. Its platform-independent design provides an easy interfacing to virtually any experimental environment. The system performance is demonstrated with the isotope depletion of a complex polyoxyalkeneamine distribution (produced by matrix assisted laser desorption/ionization) and the isolation of a single isotope from the charge-state distribution of cytochrome c (produced by electrospray ionization) in FTICR-MS measurements.

5.1 Introduction

The theoretical considerations in Chapter 2 demonstrate that the intrinsic versatility of ion traps derives from the ability to manipulate the ions with radio frequency (rf) fields. For example, frequency-selective excitation makes it possible to detect, eject or kinetically excite ions of selected $m/z$ ratios. Marshall and co-workers developed the concept of Stored Waveform Inverse Fourier Transform (SWIFT) excitation, inspired by “tailored” broadband selective excitation in Nuclear Magnetic Resonance (NMR) spectroscopy [121] to address this need of frequency-selectivity. Their method no longer constructs the waveforms in the time domain. Instead, the desired post-excitation cyclotron radius spectrum is arbitrarily specified in the frequency domain. The corresponding time domain excitation waveform is subsequently calculated by inverse Fourier transformation of that spectrum. The immediate problem in this approach is imposed by the potentially wide frequency range involved in FTICR-MS broadband excitations, because the initial time domain ex-
citation power is determined by the integrated magnitude spectrum. In an FTICR-MS instrument equipped with a 7 T magnet for example, the cyclotron frequencies of ions in the mass range $10^3 < m/z < 10^4$ cover the frequency range from $10^4...10^7$ Hz. Excitation of ions to a cyclotron radius of 1 cm over this frequency range would require rf excitation powers of $10^4 - 10^5$ V. In practice, generation of such a waveform would be equivalent to impulse excitation, and spectral resolution would be lost. Modern SWIFT algorithms deal with this amplitude problem by specification of a quadratic continuous phase spectrum next to the arbitrary magnitude spectrum, which effectively spreads the excitation power over the duration of the waveform [60, 122–125].

Naturally, it is not possible to generate SWIFT excitation signals with (programmable) frequency generators. Since the first SWIFT experiments, which were carried out using home-built electronics [121], PC-based systems [126] and, more recently, commercially available arbitrary waveform generators (AWG) [127, 128] have been used to convert the digital data to analog excitation signals. These systems typically consist of a static memory bank of 16 kword to 1 Mword and a digital-to-analog converter (DAC) with a precision of typically 8 to 12 bit. The sample rates vary from 1 to 20 MHz, which corresponds according to the Nyquist criterion to maximum output frequencies of 0.5 to 10 MHz. Although such system properties already allow for a wide range of experiments, the flexibility in designing experimental event sequences is still rather constrained. Given that a single SWIFT waveform can easily occupy up to a few Mword of memory, it is generally not possible to use several SWIFT waveforms in a single sequence without transferring new data to the waveform memory. The latter brings about a dead time of typically several seconds in between subsequent waveforms.

To overcome these limitations, we have developed a novel AWG system dedicated to ion excitation in ion traps. Its user interface is hosted by a UNIX-based workstation, which is also used to calculate the digital waveforms. This configuration possesses unique features based on its unprecedented solid state memory size of 192 Mb, its real-time properties and its stand-alone operation. For example, the size and number of waveforms used in a sequence is practically unlimited because virtually all required waveforms can be stored in the system’s memory prior to the start of an experiment. Thus, no limitations are imposed by the rate and the volume of the data transfer to the system. In addition, the AWG system can be connected to any experimental apparatus, because the interface merely consists of a trigger and an analog signal line. The required output sequences are produced by programming the sequencing software that runs on the local AWG processor.

In this chapter, the details of the in-house designed electronics and the functionality of the in-house developed software are described. The performance of the novel AWG system is demonstrated with FTICR-MS measurements. The results of isolating a number of components of a synthetic polymer molecular weight distribution by simultaneous ejection of all other components and isolating a multiply charged protein isotope illustrate the versatility and frequency selectivity of the system.
5.2  Design

5.2.1  General Design

Figure 5.1 shows the block diagram of the electronics that are of importance for the AWG-based FTICR-MS experiments. The dashed lines indicate the physical separation of the newly constructed AWG system, its host computer, and the FTICR-MS instrument with its relevant components, which run as “stand-alone” systems. Details on the in-house developed hardware and software will be presented below. Here, the basic operating principles are described briefly.

The arbitrary excitation waveforms are calculated on the host computer and are subsequently transferred to the memory of the AWG system. Also AWG event sequences are constructed on the host computer using waveforms stored in the AWG system. Delays, triggers, and loops can be added to these waveforms. After loading the event sequence into the local processor, experiments can be started. During experiments, the AWG system is exclusively waiting for external triggers from the FTICR-MS control electronics to generate analog signals from the digital data. The order of the waveforms, delays, and triggers are controlled by the local processor. If necessary, it also switches hardware settings (e.g., the bandwidth and the attenuation level) in between subsequent waveforms. The output of the AWG system is connected to the FTICR-MS instrument, where it is amplified and split into two 180° out-of-phase channels to drive the excitation plates of the ICR analyzer cell.

5.2.2  Description of the AWG hardware

The backbone of the AWG system is the VME instrumentation bus. The local processor (25 MHz VME processor board 68040, Motorola) is its bus master and is booted automatically via the Ethernet using the host computer (Indigo Irix 5.3 UNIX-based workstation, Silicon Graphics, Mountain View, CA) as boot site. It configures the various system components prior to the generation of a waveform. The waveforms are loaded from the host computer via the BIT3 Bus Bridge connection to the VME bus into the memory boards of the AWG system (3 × 64 Mbyte, Chrislin Industries Inc., Westlake Village, CA). The waveforms have to be encoded in a special format, in which every three 32 bit words contain 8 samples of 12 bits each (corresponding to the resolution of the DAC). According to this format, the three nibbles (4 bit entities of data) composing each of the eight samples are divided over the three 32 bit words. In turn, each set of three 32 bit words is divided over the three memory boards and stored at the same memory location. This data format is introduced to obtain a sustained sample rate of maximally 20 MHz next to a maximum cycle rate of 10 MHz at the VME Subsystem Bus (VSB bus), as will be shown later. An additional advantage is the optimal usage of the available memory (the packed format allows for 128 Mword sample storage in the AWG system).

Sample processing hardware was designed in-house to read the data describing the waveforms from the memory modules and to convert them into a continuous stream of
Figure 5.1: Block diagram of the electronics for AWG-based FTICR-MS experiments.
decoded 12 bit samples. The sample processing hardware consists of three FIFO (First In First Out memory) boards, which are based on a 2 kbyte FIFO buffer, and a FIFO controller board. Communication between these boards is realized via an additional control bus. The FIFO boards are connected to the memory boards via independent VSB side busses. This allows reading of the data in blocks of 128 words (that is, 512 bytes) from all memory boards simultaneously, at the maximum VSB cycle rate of 10 MHz. The start and finish address of a waveform as well as its bandwidth are loaded from the local processor into the FIFO controller board prior to the generation of a waveform. The control board starts the FIFO boards on the rising edge of an external trigger signal to extract in parallel nibbles from the FIFO buffers at a continuous rate of twice the waveform’s bandwidth. This rate is governed by the 40 MHz internal clock of the FIFO controller board and can be adjusted by dividing the 40 MHz by powers of two in the range from 156 kHz to 20 MHz. The FIFO boards present the extracted nibbles in parallel to the data bus, jointly composing the original decoded 12 bit samples. During this process, a new VSB data transfer is initiated by the FIFO controller to replenish the FIFO buffers when 512 bytes have been freed. This continues until the entire waveform has been processed.

The stream of samples produced by the sample processing hardware (at the rate of twice the bandwidth) is interpolated once by a digital FIR (Finite Impulse Response) filter, producing a stream of samples at the rate of four times the bandwidth. Conversion to an analog signal is realized by a 12 bit DAC (Burr Brown). The FIR filter and the DAC are operated at a clock signal of four times the bandwidth, which is received from the internal clock of the FIFO controller board. Frequency components outside the bandwidth are removed from the excitation signal by the combination of a tunable and a 10 MHz low-pass filter. The dynamic range of the excitation signal can be modified by adjusting the attenuator (0-60 dB in steps of 4 dB). Additional fine-tuning of the dynamic range is available by adjusting the reference voltage of the DAC via an additional reference DAC, which sets the maximum output voltage of the 12 bit DAC. The settings of the tunable filter, the attenuator, and the reference DAC are initialized by the local processor via the in-house designed digital input-output module. Finally, the output of the AWG system is connected to the transmitter of the FTICR-MS instrument via a relay switching circuit, which allows for fast switching (dead time of the circuit is approximately 100 μs) between AWG excitation signals and other excitation signals (e.g., the standard Bruker programmable frequency synthesizer) during experiments. This relay is operated on trigger pulses from the FTICR-MS control electronics such that the AWG output is connected to the transmitter if the trigger input is high.

After completion of the waveform, the local processor initiates the next task. This can be a next waveform following a variable time delay or a new trigger from the FTICR-MS control electronics. Alternatively, if the AWG event sequence is completed, it can be restarted automatically. The latter facilitates for example signal averaging over multiple experiments. Usually, various hardware parameters have to be reset in between subsequent waveforms, causing a dead time of approximately 1 ms.
5.2.3 Description of the Software

Software was developed in-house for the calculation of waveforms, the construction of AWG event sequences, the communication between the host computer and the AWG system, and the task managing within the AWG system. This software consists of two elements: TasMan that runs under the real time operating system LynxOS on the local processor, and XAWG that runs under UNIX on the host computer.

The TasMan program controls the operation of the AWG electronics. For this purpose, it maintains a database of the waveforms that are stored in the systems memory and the associated hardware settings. It communicates with the host computer via the Ethernet in a server-client relationship. This communication line is used to receive a second database, which describes the composition of the AWG event sequence, and the commands to start and finish executing this sequence. During the sequence, the TasMan program configures the various hardware parameters when appropriate. In this way, it allows for real-time switching between subsequent pulses within 100 µs.

The XAWG program assists in the design and calculation of (SWIFT) waveforms, the design of AWG event sequences, and the communication with the AWG system. It is compatible with the data format used by the Bruker software XMASS. Loading a measured FTICR-MS transient signal into XAWG automatically sets defaults for various variables in the calculations. For example, the bandwidth for the excitation, the size of the data set, and the calibration constants for the transformations between the frequency and the mass domain are copied from the experimental parameters. After Fourier transformation of the transient, an excitation profile is constructed on the basis of the mass spectrum in the graphical user interface with standard functions. These include rectangular magnitude versus mass segments (determined by a center mass, a mass width and an excitation radius), and sinusoids in the mass domain (useful in two-dimensional FTICR-MS experiments [129]). The excitation profiles are saved in terms of the building objects, which allows for readily adjusting them at all times. Smoothing of the excitation profile by convolution of the spectrum with a rectangular pulse in the frequency domain [60, 124] is available to minimize wiggles (so-called Gibb’s oscillations) in the actual magnitude spectrum of the calculated waveform. Subsequently, the desired post-excitation cyclotron radius as a function of mass is converted to excitation voltage magnitude versus frequency. This spectrum is used to calculate the quadratic phase versus frequency function for an optimal spread of the excitation power in the SWIFT waveform with an algorithm that is based on the methodology developed by Guan and McIver [124]. Inverse Fourier transformation of the magnitude and phase spectra yields the SWIFT excitation waveform. Finally, the ”true” excitation spectrum is examined by zero-filling and Fourier transformation of the SWIFT waveform.

In addition to the SWIFT waveforms, it is also possible to create the traditional time-domain excitation waveforms. For narrow band excitation, the single-frequency (burst) excitation (a sinusoid with fixed frequency and amplitude) is available. Broad band excitation can be performed using the chirp excitation (a constant-amplitude sinusoid whose instantaneous frequency increases linearly with time over a given frequency range).
The amplitude uniformity of the excitation spectrum produced is, particularly in the case of SWIFT waveforms, directly dependent on the accuracy of the digital-to-analog conversion [130]. It is therefore advantageous if the maximum amplitude of the waveform corresponds with the dynamic range of the DAC. In general, this is automatically achieved in the calculation of the optimal phase function according to the SWIFT algorithm of Guan and McIver [124]. However, if the duration of the excitation waveform is determined by the required resolution rather than the required total excitation power, a substantial mismatch may occur. Therefore, all excitation waveforms are normalized to fit the -2048 ... 2047 range corresponding to the 12 bit resolution of the DAC. The desired excitation power is obtained by adjusting the attenuator and the reference voltage of the DAC to the values that are calculated from the normalizing constant.

The normalized waveforms are written to disk in the special data format compatible with the AWG hardware. The associated hardware settings are attached in a header. A loader is implemented in the XAWG program to load these waveforms to the memory of the AWG system and the hardware settings to the TasMan program. The final part of the XAWG program, the sequencer, acts as the user interface to the AWG system. It retrieves the database describing the waveforms available in the AWG memory from the TasMan program and allows the user to design the AWG event sequence.

The system’s flexibility in constructing the most versatile experiments is illustrated on the basis of the exemplar AWG sequence that is depicted in Figure 5.2. Also an external FTICR-MS trigger signal and the generated AWG output are included in the figure. Its practical relevance is that it contains all excitation events that are required for FTICR-MS experiments in which collision-activated dissociation (CAD) [131–133] of ions is achieved by application of sustained off-resonance irradiation (SORI) [134]. Briefly, it comprises the isolation of the precursor ions, the alternating acceleration and deceleration of these ions between a starting radius and a larger radius by an off-resonance burst excitation (typically, \( \Delta f \) is varied from \( 10^2 \) to \( 10^3 \) Hz), and finally, the broad band excitation to produce a detectable signal for all precursor and fragment ions.

The sequence shows that subsequent waveforms can be separated by either external triggers or internal delays. It is also seen that the trigger signals remain high for the entire part of the sequence that they initiate. This is required because otherwise the AWG system will abort the sequence to prevent that other tasks managed by the FTICR-MS instrument (e.g., the detection event) unintentionally coincide with the AWG excitation events.

Additionally, the sequence exhibits that any part of it can be repeated several times. The main loop in the example is generally used to perform experiments in which the results of several measurements are averaged. The second loop is used to produce a single-frequency excitation signal from a waveform consisting of one period of a sinus. This is only possible by virtue of the systems capability of real-time and instantaneous pulse switching. It is obvious that this saves considerable amounts of memory compared to storing the complete waveform. It should be noted however that this is only possible if an integer number of periods of the sinus can be fitted into the block size of the data transfer between the memory modules and the FIFO buffers (that is, \( 128 \times 8 \) samples).
Figure 5.2: Example of an AWG event sequence that illustrates the flexibility provided by the AWG system for the construction of experiments. Also shown is the AWG output that is generated by this sequence if the depicted external FTICR-MS trigger signal is applied to the system.

This condition can be written as:

$$128 \times 8 f_{exc} = n f_{sample}$$  \hspace{0.5cm} (5.1)

Here, $n = 1, 2, 3, \ldots$, $f_{exc}$ is the excitation frequency, and $f_{sample}$ is the sample rate. If this condition is not met, a discontinuity will appear every time that the block of data is repeated.

### 5.3 Experimental

#### 5.3.1 Instrumental

The FTICR-MS experiments were performed on the modified Bruker-Spectrospin (Fällanden, Switzerland) APEX 7.0e FTICR-MS. The instrumental layout and experimental procedures are described in detail in Chapter 2. Briefly, the MALDI experiments were carried out in the MALDI/EI source by depositing the samples on the stainless steel tip of a direct insertion probe and introducing this tip into the external ion source via a vacuum lock. Ions were produced by irradiating the samples with the 337.1 nm wavelength beam
of a nitrogen laser and they were subsequently transferred into the analyzer cell by electrostatic ion optics. The ESI experiments were performed in a newly in-house constructed source, which is described elsewhere [135]. In the ESI geometry, a syringe pump was used to deliver the samples via a deactivated fused silica capillary to a stainless steel spray needle at a flow rate of 0.1 mL/hr. Positively charged electrosprayed droplets that were generated by applying ~ 3000 V to the spray needle, were accelerated towards a heated stainless steel capillary at 100 V. Subsequently, they were focused through a skimmer for removal of the excess neutrals and guided towards the electrostatic ion optics using an rf-only quadrupole.

After trapping of the ions, a TTL trigger pulse was applied to the relay switching circuit in the AWG system to connect the AWG output to the transmitter. This was followed by a 3 ms time delay to allow the relay to settle. A second TTL trigger pulse was applied to the AWG to initiate the generation of the loaded excitation waveform for ejection of ions of unwanted \( m/z \) values from the cell. After lowering these trigger signals, the ions were excited to a high and coherent cyclotron orbit by a series of single-frequency excitations generated by the Bruker programmable frequency synthesizer (frequency shifted over the excitation range in steps of 20 \( \mu \)s duration with an increment of ~ 5.2 kHz). This coherent motion was detected at an acquisition rate of typically 500 kHz into 128 kbyte data points. The resulting time domain signal was zero-filled to 256 kbyte, followed by discrete Fourier transformation and magnitude calculation and finally transformed to a mass spectrum.

### 5.3.2 Sample Preparation

The samples investigated are Jeffamine D2000 and cytochrome c and are both commercially available. The Jeffamine sample was obtained from Texaco Chemical Company and measured by means of MALDI-MS. According to the manufacturers data sheet (SC-024 102-0411), the Jeffamine D2000 is an amine-terminated polypropylene glycol with the general structure \( \text{H}_2\text{N}-(\text{C}_2\text{H}_6\text{O})_n-\text{C}_3\text{H}_6\text{NH}_2 \) and an average molecular weight of 2000 u. Previously published FTICR-MS investigations on this sample [112] revealed that at least three additional polymer series are present in the D2000 sample at lower masses due to contamination, early termination reactions during polymerization, or oxidation. The matrix in the MALDI experiments was 2,5-dihydroxybenzoic acid (DHB) from Sigma Chemical Co. (St. Louis, MO). The Jeffamine was prepared for MALDI measurements by mixing a 1 M matrix solution in ethanol with an approximately 10 g/L Jeffamine solution in ethanol to give an average molar ratio matrix:analyte = 1000:1. This mixture was electrosprayed onto the stainless steel MALDI probe tip (Section 3.2.2). Approximately 0.1 mL analyte/matrix was consumed during sample deposition.

Cytochrome c of horse heart was measured by means of ESI-MS and was purchased from Aldrich (Milwaukee, WI). Cytochrome c was sprayed from a ~ 1 \( \mu \)M solution with a solvent composition of 68:30:2 MeOH:H\(_2\)O:HAc. The average molecular weight of cytochrome c was calculated to be 12360.18911 u on basis of its amino acid sequence.
(elemental composition $C_{526}H_{842}N_{144}O_{152}S_4$) and the elemental composition of its heme ($C_{34}H_{80}N_4O_4Fe_1$ [137]). It should be mentioned that the mass of two hydrogen atoms was subtracted for each of the two bonds between the heme and the thiol groups of the cysteine residues and that the acids in the heme group were assumed to be protonated.

5.4 Testing the Performance by Mass-Selective Ion Isolation in FTICR-MS

MALDI-FTICR-MS spectra of Jeffamine D20000 shown in Figure 5.3 were recorded by summing 100 consecutive laser shots at a trapping delay of 1000 $\mu$s to get a maximum signal-to-noise for the main polymer series.

The spectrum in Figure 5.3 A was recorded without actively ejecting ions from the analyzer cell and hence reflects the ion population initially trapped. It shows that two polymer series are present in the mass range $m/z$ 1000 to 2400. Both correspond to the amine-terminated polypropylene glycols at equidistant intervals of 58 u. The most abundant series was cationized by sodium attachment, and the other series by protonation. The inset with expanded mass scale shows that the naturally occurring isotopes of the component molecules are resolved and that the dominant series originates from sodium cationization, whereas the series at lower abundance is formed by protonation. The ions observed in the mass range below $m/z$ 1200 correspond to the additional polymer series that are present in the sample [112]. The abundance of these series is however highly underdetected because trapping was not efficient in this mass range due to the flight-time-induced mass discrimination (Section 3.4.1).

The spectra in Figure 5.3 B and 5.3 C show the effect of ejecting all ions within the mass range $m/z$ 1200-2000 that are not part of the dominant sodium cationized polymer series between the components at $m/z$ 1316 and $m/z$ 1896. This was realized by using SWIFT waveforms with a comb-like excitation profile as is indicated by the dashed lines in the bottom spectrum. The bandwidth of these excitation waveforms was 2.5 MHz, the size 2 Mwords, and the duration ~ 0.2 s. The mass width of the notches in the applied excitation profile was 6.0 u for the isolation of the entire isotopic clusters in the middle spectrum and 1.0 u for the isolation of the monoisotopic peaks in the upper spectrum. The attenuation level of the SWIFT waveform was optimized to the level that ions are just ejected after completion of the waveform. This was done by omission of the broad band excitation and subsequently monitoring the increasing signal from the protonated polymers in a series of measurements in which the attenuation level was decreased (data not shown). The attenuation level at which this signal suddenly disappeared was selected to perform the measurements presented here. At this level, no signal was observed from polymers inside the notches. The upper two mass spectra demonstrate that this efficiently removes the protonated polymer series within the ejection mass range from the analyzer cell. Moreover, the expansions of the mass scale prove that no severe power leakage into the notches occurs because peak intensities and isotopic patterns are not significantly changed. Finally, the efficient isolation of the monoisotopic peaks in the upper spectrum
Figure 5.3: MALDI-FTICR-MS spectra of Jeffamine D2000, illustrative of the capabilities of the novel AWG system. The top spectrum (A) was recorded without the application of isolation waveforms. The dashed lines indicate the comb-like excitation profile of the SWIFT waveform applied to obtain the remaining spectra (B & C). The mass width of the notches in the excitation spectrum was 6.0 u (B) and 1.0 u (C), respectively. The insets illustrate the effect of the isolation waveforms.
is indicative of a frequency selectivity of better than 30 Hz in the isolation event. This value is based on the frequency difference between the isotopic peaks of the 32-mer (the monoisotopic peak at $m/z$ 1954 corresponds to 54929 Hz, the first isotopic peak at $m/z$ 1955 to 54901 Hz).

The frequency selectivity in AWG excitation events was investigated in more detail by ESI-FTICR-MS on cytochrome c. The mass spectrum recorded by broad band excitation using the Bruker frequency synthesizer is shown in Figure 5.4 A. An abundant

![Figure 5.4: Ultrahigh frequency selective SWIFT isolation with the novel AWG system: A) broad band ESI-FTICR-MS spectrum of cytochrome c covering the entire charge-state distribution that ranges between 11+ and 18+; and (B) mass spectrum following the broad band SWIFT waveform shown in Figure 5.5. The insets demonstrate the successful isolation of a single isotopic peak.](image)

distribution of ions is observed in which each ion corresponds to a different charge state of the molecular species. The expanded mass scale shows that the mass resolution (here, $(m/dm)_{50%} = 24400$ at $m/z$ 824.97) is sufficient to resolve the $^{12}$C and $^{13}$C isotope peaks for the individual charge states. The number of isotope peaks appearing within a single mass unit was determined to identify the number of charges on the ion [138]. In this way it was found that the charge state distribution in Figure 5.4 A ranges between 11+ (around $m/z$ 1124.8) and 18+ (around $m/z$ 687.8). The ionizing species was identified by determining the average $m/z$ ratio for the isotopic cluster shown in the expanded
mass scale and multiplying it with the charge state \( z = 15 \). Comparison of the result (12374.21 u) with the theoretical mass calculated on basis of the cytochrome c amino acid sequence (12360.19 u) demonstrates that these ions were produced by proton attachment. However, this leaves a discrepancy between the measured and the calculated mass. This is illustrated by Table 5.1, which lists the molecular weights determined from different charge states. In the mass determination it was taken into account that the iron in the heme of cytochrome c is oxidized and carries three charges [137], and it was assumed that the remaining charges were added by the attachment of protons. It is seen that the experimentally determined values differ by typically 2 u from the theoretically calculated one. The deviant values for the 11+, 17+, and 18+ charge states are attributed to their poor S/N ratio. The difference of 2 u is significantly larger than the expected accuracy in the mass determination (which is estimated to be \( \pm 1 \) u). Moreover, it is in agreement with results presented in other publications in which the charge of the oxidized iron was not considered. For example, Banks et al. determined a molecular weight of 12359.13 u from their ESI spectra with the assumption that all charges were the result of proton attachment [139]. Addition of the mass of three hydrogen atoms yields 12362.15 u. Beavis et al. determined with MALDI-ToF [140] a mass of 12360.1 u for the singly charged molecular ion, which gives 12362.1 u. Therefore, it is presently assumed that this discrepancy in mass is not produced by artifacts in the measurements but that the elemental composition of cytochrome c is not unambiguously determined by its amino acid sequence.

In addition to the main distribution, also ion series are observed that correspond to molecular masses that are approximately 60.7 u and 123.2 u larger than the protonated molecular species. These series result from deterioration of the sample due to the storage conditions of the sample solutions and are not observed when fresh samples are studied. For example, an increase in mass of 60.7 u can be explained by the attachment of acetate.

The inset in Figure 5.4 shows an expansion of the mass scale around the 15+ charge

Table 5.1: Molecular weights of horse heart cytochrome c, determined from the different charge states in the spectrum of Figure 5.4 A.

<table>
<thead>
<tr>
<th>Charge state</th>
<th>molecular weight</th>
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<tbody>
<tr>
<td>18+</td>
<td>12361.68</td>
</tr>
<tr>
<td>17+</td>
<td>12361.90</td>
</tr>
<tr>
<td>16+</td>
<td>12362.13</td>
</tr>
<tr>
<td>15+</td>
<td>12362.11</td>
</tr>
<tr>
<td>14+</td>
<td>12362.17</td>
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<td>13+</td>
<td>12362.19</td>
</tr>
<tr>
<td>12+</td>
<td>12362.29</td>
</tr>
<tr>
<td>11+</td>
<td>12362.50</td>
</tr>
</tbody>
</table>
state. The circles indicate the theoretical exact masses and normalized relative abundances in the isotopic pattern. This pattern was calculated for the elemental composition of cytochrome c plus two additional hydrogen atoms. The position of the monoisotopic peak and the peak corresponding to ten $^{13}$C isotopes are indicated. It is seen that the calculated and the measured patterns match. The deviations between the exact masses and the measured masses were within 0.015 u around the center of the pattern.

The expanded mass scale also reveals the close isotope spacing for the 15+ charge state. The difference in $m/z$ ratio between adjacent isotope peaks in this charge state is equal to 0.067 u. This corresponds to cyclotron frequency differences of less than 10 Hz at an average cyclotron frequency of 130.2 kHz. Isolation of a single isotope peak from this cluster is evidently challenging and an excellent test for the system’s frequency selectivity. This was achieved by tailoring a 1 Mword broad band SWIFT waveform to eject all ions from $474.95 < m/z < 824.95$ and from $825.02 < m/z < 1375.02$ and to leave ions from $824.95 < m/z < 825.02$ unaffected. The time domain waveform (312.5 kHz bandwidth and 1.68 s duration) is depicted in Figure 5.5. Included in the figure is its actual excitation profile, which was obtained by Fourier transformation of the SWIFT waveform after zero-filling to 2 Mword. The inset reveals the ultra-high resolution of the mass notch at $m/z$ 824.865 and predicts a theoretical frequency selectivity of better than

![SWIFT Isolation Waveform](image)

![Swift Excitation Profile](image)

**Figure 5.5:** Broad band SWIFT waveform and its actual excitation profile used to isolate a single isotope peak from the broad band ESI-FTICR-MS spectrum of cytochrome c. The excitation profile was specified to eject ions from $474.83 < m/z < 824.83$ and from $824.90 < m/z < 1374.90$. The inset illustrates the ultra-high resolution of the notch in the excitation profile.
The mass spectrum recorded after application of this ejection waveform is shown in Figure 5.4 B. It demonstrates that all unwanted ions were successfully removed from the cell. Moreover, comparison of the resolution and the S/N ratio of the peak at \( m/z \) 824.85 before and after isolation (see the expanded mass scales in Figure 5.4 A and 5.4 B) indicates that the isolated ions remain unaffected.

Finally, the ejection efficiency around the borders of the isolating notch was investigated in more detail by performing similar experiments in the high-resolution (heterodyne) detection mode. Figure 5.6 A and 5.6 B show the high resolution spectrum without the application of any SWIFT isolation waveforms and one that follows the SWIFT waveform shown in Figure 5.5, respectively. The expanded mass scales illustrate clearly the increased resolution in comparison with the broad band spectra (\((m/dm)_{50\%} = 97000\) at \( m/z \) 824.85). Also some additional low-intensity peaks are observed in the high resolution mode, which were not clearly observed in the broad band spectrum in Figure 5.4 A. These are indicated in the spectrum and correspond to partial sodium cationization (for example, [M+Na+1H]^{10+} instead of [M+12H]^{10+}), and oxidized cytochrome c. Fragment ions with eight charges attached are observed around \( m/z \) 817.8. The mass spectrum

![Image](image-url)
recorded after application of the SWIFT ejection is shown in Figure 5.6B and demonstrates that again all unwanted ions were successfully removed from the cell, whereas the isolated ions remain essentially unaffected.

The frequency selectivity was quantified by performing a series of similar experiments in which the center of the notch was shifted in steps of 0.01 u (corresponding with 1.4 Hz) from $m/z = 824.95$ to $m/z = 825.05$. In these experiments it was observed that a shift of 0.03 u in the center of the notch corresponds to the difference between efficient ejection and efficient isolation. We therefore conclude that the frequency selectivity at the boundaries of the isolation notch was approximately 4 Hz.

5.5 Summary and Conclusions

The design of a novel arbitrary waveform generator dedicated to the excitation of ion motions in ion traps has been described. FTICR-MS experiments have been performed with this generator in which monoisotopic components from the molecular weight distribution of Jeffamine D2000 and single isotopes from the charge-state distribution of cytochrome c have been successfully isolated in the analyzer cell. Based on these measurements the frequency selectivity allowed by the system hardware is estimated to be better than 4 Hz. This accuracy is however worse than the resolution in the calculated waveforms ($\approx 1$ Hz). It is presently believed that this is due to off-resonance excitation and not due to frequency instabilities in the AWG hardware. Future experiments based on longer isolation waveforms are envisaged to prove this.

By virtue of the AWG's large memory size and its flexibility in constructing output sequences, the system is ideally suited for complex FTICR-MS experiments such as SWIFT-based two-dimensional mass spectrometry [129] and multistage tandem mass spectrometry. Its stand-alone operation and easy interfacing make the AWG compatible with virtually any experimental apparatus.

Finally, new instrumental developments are currently undertaken, which will make it possible to use the memory of the AWG system for data acquisition too. The main advantage of this development is that it will become possible to acquire broad band transients for several seconds. This implies that mass resolving powers that are presently only available in heterodyne mode will also become available in broad band spectra over the entire mass range.