

# Mass Spectra Based Fraction Collection of Proteins and Peptides - Cheap, Quick and Dirty

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## INTRODUCTION

In recent years, the drug discovery process has made great strides due to the advancement of technology in instrumentation for use by chemical and biochem scientists. Identifying active products and understanding biological mechanisms are an essential step in the drug discovery process. Isolating and purifying pharmaceuticals as well as intact proteins and digests of proteins is integral to the discovery of the needed cures for dread illnesses and diseases.

Many methods exist for the purification of intact proteins and digests of proteins. Page, "Spot" and ultra filtration techniques are widely used. Chromatographic techniques like ion-exchange, chelate, heparin and dye chromatography are also used. In each case, after separation, it is common for evaluation to be done by mass spectrometry for final determination of purity.

One chromatographic method, liquid-phase chromatography, can be coupled with detectors of some sort (selective or non selective) to provide repeatable and accurate time based separation. In fractionation, entire chromatographic peaks (0.7 to 1.5 minutes wide) are "caught" for further evaluation or use. One big advantage of liquid chromatography is that it can be directly coupled to mass spectrometry. With this technique, Mass Based Fractionation, mixtures can be separated and sent directly into the inlet of a mass spectrometer providing immediate, on-line, mass selective spectral information.

This paper discusses a software product that has been developed that ties to a commercially available mass spectrometer (HPLC/MS) that can enable the chemist to take the first steps in isolation and purification, at a very low cost.

## DISCUSSION

In the past, purification of products has been widely done by UV detection of the output from a high performance liquid chromatography (HPLC) system. The chemist would set up an HPLC run, inject an amount of product mixture, and through an analytical, semi-preparative or preparative column by way of UV, "catch" the peaks in individual containers (vials, tubes, cells, bottles, jars, etc...) as they come off the column. Then, each fraction is analyzed by mass spectrometer, to verify the makeup and purity. The analyst would often catch before and after fractions, to aid in the determination of the purity of the desired fraction, and for recovery purposes (catch what's left) for later use.

Some automation of UV fraction collection has occurred over the years by manufacturers of robotic instruments like the Foxy 200 and Foxy Jr. fraction collectors (Isco, Inc. Lincoln NE). Other manufacturers include: Gilson, Spectrum Chromatography, Eldex, Agilent, Buchi, Waters, and others. Using the analog signal from the UV detector, the unit can be programmed to collect fractions into test tubes or other containers.



Years later, mass based fraction collection, using Atmospheric Pressure Ionization (API) techniques, typically electrospray, became available. Expensive and elaborate mass spectrometry systems, including well plate auto samplers using the 96 & 384 well format start by delivering a sample on column and by mass spectrum, are fractionated back into new wells. Then, the fractions can be automatically analyzed for content and purity. These systems typically cost \$250,000 or more and can be quite complicated to run. The complexity generally centers around the well plate autosampler, but can also be attributed to the fact that some vendors require a second HPLC pump to provide "make up" flow, after a split in the flow path. All in all, very expensive.



## SOFTWARE INTRODUCTION

A software product has been developed that ties to a commercially available mass spectrometer (Agilent 1100 LC/MSD) that can enable the chemist to take the first steps in isolation and purification, at a very low cost. This software product, Mass Manager (© 1998 CSS Analytical Co. Inc. Shawnee, KS 66218) evaluates the spectra, being collected by the mass spectrometer, and alerts the user to a specific  $m/z$  (mass, Dalton, or mass to charge) being seen at the inlet. When the green light comes on the screen, flip a valve or move the end of the tubing to a clean vial. With this substantially manual process, mass based fractionation can be accomplished without great expense. This software solution is ideally suited for graduate or undergraduate studies, and start up labs with limited budgets.

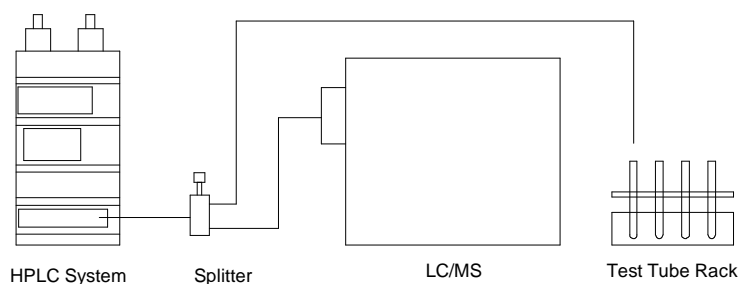
## INSTRUMENT DETAILS

The software presented was written for use with the Agilent 1100 LC/MSD benchtop single quadrupole mass spectrometer with Electrospray (ESI) Ionization Source. The Mass Spec system also includes an Agilent 1100 HPLC modular system with quaternary pumps, vacuum degasser, thermostated column compartment, 100 vial autosampler and variable wavelength detector. The system was controlled by the Agilent Multi-Technique Chemstation on a Personal Computer running Windows 2000 via LAN connection and BootP Server. The 1100 LC/MSD was enhanced by the CSS Analytical Co. Inc. G1946css+ High Sensitivity Upgrade.

## SETUP DETAILS

Typical fractionation systems start with an HPLC system and a column. The column can be analytical, semi-preparative or preparative in nature, which basically defines the size of the column and the capacity or flow rate of the mobile phase (mp). The column must be matched to the HPLC pumps. Typically, analytical columns allow for a flow of mp at a rate of 0.2 ml/min to no more than 4 or 5 ml/min. Semi-Preparative columns can accommodate flow rates from 10 to 30 ml/min and preparative columns can handle higher flow rates. Naturally, size of the column also defines the amount of sample that can be "loaded". This is the total amount of injected analyte (in ng, ug, or mg) allowed on the column, of which, a portion will be caught (fractionated) and saved.

The HPLC system may or may not include a detector of some sort. Typically a variable wavelength or diode array detector is included. Since the system will fractionate by "mass" the detector is not needed. However, many ms operators rely heavily on their UV detectors in order to "see" the peaks coming. Often times, chromatography is worked out off-line, and the UV trace of a given experiment is known. The UV detector is a very appreciated aide to developing the ms parameters required to accomplish ionization and detection inside of the mass spec.



Once the analyte has been injected into the HPLC system, and optionally passed through the HPLC detector, the stream is split with one side of the split going to the mass spec and the other side of the split going to the fractionation device, which, in this case is a manually controlled.

The split ratio of ms to waste (and fractionation tubes) is typically on the order of 1:10 or 1:15. Remember, as you split the stream, more to fractionation and less to mass spec, the less amount of analyte will be going to the mass spec, and therefore the abundance trigger is lessened. The obvious goal is to balance the split between the mass spec and the fraction collector, in favor of collecting as much material as possible. Flow rates also play an important part, a sufficient flow must be sent to the mass spec sprayer for efficient ionization. What ever sample concentration and flow rate the mass spec needs, must be delivered to the ms – after the split. Splitters used in this method can vary from very inexpensive, to home made, to highly precise. Inexpensive (but very good) splitters can be purchased from Upchurch Inc, (Oak Harbor, WA). The model P-450, all plastic, costs \$40 on the low end and the P-470, with stainless or titanium needle and graduated adjustment will cost \$240. Splitters with fixed and very precise ratios are available from LC Packings (San Francisco, CA) can cost several thousand dollars.

## SOFTWARE OPERATION

Software operation is quite simple. It substantially consists of two executables and a macro. One executable is a “configuration” file, and the other executable is the program that runs in background that “parses” the information from the mass spec and alerts the user that a fraction is coming. The macro is a “start-up” macro, which ties the external executable to the mass spec software.

To prepare for fraction collection, you must create fraction collection methods. Fraction collection methods work with the standard ChemStation data acquisition methods. The data acquisition methods also require very minor modifications. Once the methods are ready, you can run samples just as you would for any other analysis. Some sample information must be entered for each sample from which fractions will be collected, in addition to the sample information you would normally enter. Fractions can be collected from single samples or from sequences of samples. Fractions can be collected from selected samples within a sequence of samples.

Fraction collection methods are different and separate from the standard ChemStation data acquisition methods. Fraction collection methods (the first executable program, Fractcfg.exe) define the mass window around the target mass and the abundance level that triggers fraction collection. They also define adducts that are used to trigger fraction collection.



Data acquisition methods must be modified before they can be used for fraction collection. A pre-run macro specified in the Run Time Checklist starts the fraction collection program when the method is run. Every method to be used with fraction collection must be modified and saved individually. The target mass and fraction collection method to be used must be entered in the sample information/comments field for each sample from which fractions will be collected. Because the target mass is specified here instead of in the fraction collection method, the same method can be used with different target masses for different samples. The target mass and fraction collection method are not stored as part of the method. They must be re-entered each time a sample is run. A pre-run macro is also required to start the fractionation macro, thus making the tie between the above “configuration” executable and the mass spec software. Setting up data acquisition methods are quite easy. Setting up sequences (up to 100 vials) is also quite easy to do, allowing for automated operation.

Once the mass spec run is started, the pre-run macro starts the fractionation executable (Fract2.exe), and the injection process continues. If the run is set up for manual injection, the user will be prompted to make an injection. If the injection requires the autosampler to pick up a vial, then it will proceed. Once the injection starts, the fractionation executable begins its process of parsing the data coming from the mass spectrometer and alerts the user with a green light when the desired target mass (appropriate m/z, including adducts) with EIC exceeding threshold appears.

An important part of the configuration (Fractcfg.exe) not previously mentioned is the “Delay” time. This factor, is a delay that accounts for the time that the fraction needs to get from the splitter into the test tube. This delay time is completely hardware dependent. It depends upon split ratio, flow rate, mobile phase composition, tube length and tube diameter. Once the delay time is experimentally determined or calculated, the user can plug in this programmable delay factor, giving the user flexibility to allow for particular plumbing. One really neat way to determine the built in delay is to use a fluorescing die, Coumerine 504 Laser Die available from Aldridge (St. Louis, MO) in Acetonitrile. Once the physical characterizes of the system have been determined, the delay can be entered and fixed. If any changes are made, a new delay may be needed.

## CONCLUSION

A software product, written specifically for one brand of commercially available mass spectrometer, can provide the user with a unique ability to identify mass based fractions for collection by manual means, providing low cost mass based fractionation of chemical and biological compounds. An easy, quick and dirty, and don't forget cheap way to get started in compound purification – before you go to the next step, spending a lot of money.