NeoLynx Research 4.0 Application Manager User's Guide

Version 4.0

Waters part No –715000403

Micromass Part No – 6666669

6 February, 2002

CAUTION: For Research Use Only. Not for use in diagnostic procedures.
No use of this product as a diagnostic tool is implied or should be inferred.

NeoLynx Research Application Manager User's Guide

Important Notice

Micromass Limited (Manchester, UK) and its subsidiary Micromass Inc. (Beverley, MA) do not offer for sale or support the NeoLynx Research Application Manager for non-research applications within the USA.

No use of the NeoLynx Research Application Manager as a diagnostic device is implied or should be inferred.

Copyright Notice

Trademarks

Table of Contents

Conventions

The MassLynx Users Guide follows these typographic conventions

This	Represents
bold	Anything to be typed exactly as it appears
italic	Place holders for information that must be provided. For example if asked to type *filename*, type the actual name for a file instead of the word shown in italic type.
ALL CAPITALS	Directory names, filenames and acronyms.

Keyboard Formats

Key combinations and key sequences appear in the following formats.

KEY1+KEY2	A plus sign (+) between key names means to press and hold down the first key while pressing the second key. For example, "press ALT+ESC" means to press and hold down the ALT key and press the ESC key. Then release both keys.
KEY1,KEY2	A comma sign (,) between key names means to press and release the keys one after the other. For example, "press ALT,F" means to press and release the ALT key and then press the F key.

Chapter 1 Introduction

Table of Contents

Figures

Overview

The objective of this document is to explain the processes required to return a quantitative analysis of metabolites extracted from blood spots. The chemical and instrumental background on the analyses is covered in some depth, with the aim of allowing the users to develop their own analytical strategies. The principles and operation of the NeoLynx suite of processing software are discussed, and a comparison of the quantification results with classical integration methods is presented. The ultimate goal of this type of analysis is to provide a result giving the endogenous concentration of the targeted metabolites.

There are seven basic stages involved in automated analysis, which are:

1. Creation of a Project.
2. Definition of Tune page, Inlet and Scanning Method files.
3. Definition of NeoLynx Test Files.
4. Creation of a list of samples using the Sample List Editor.
5. Preparation of Samples.
6. Acquisition of sample data.
7. Processing of acquired data and production of reports using the NeoLynx Browser.

A data compendium of samples from a number of patients with inherited metabolic diseases is included Appendix A. The samples were provided by Dr Mohamed Rashed of the King Faisal Specialist Hospital and Research Center.

Introduction

In the study of metabolic diseases in newborn babies, blood spot samples are taken on filter papers soon after birth. The adsorption of blood onto filter papers has been well characterized, and it is possible to utilize a reasonably well defined amount of blood by punching a disc out of the dried filter paper.

In the analyses described in this document, the metabolites are typically extracted from the dried blood sample on the filter paper disc, chemically derivatized where necessary and subjected to analysis by electrospray tandem mass spectrometry.

The function of a tandem mass spectrometer is to:

1. Produce ions from the compounds in the sample under analysis.
2. Select PRECURSOR ions according to their mass in the first analyzer of the instrument.
3. Fragment the mass selected PRECURSOR ions, by colliding them with argon gas in the collision cell, to give PRODUCT ions - a process called COLLISION-INDUCED DISSOCIATION (CID).
4. Analyze the PRODUCT ions according to their mass in the second analyzer of the tandem instrument.

The overall process is often called MS/MS (mass spectrometry - mass spectrometry or tandem mass spectrometry) and is a highly specific way for detecting specific substances in complex mixtures

When data from samples has been acquired NeoLynx processes the results to:-

- determine the metabolite concentrations of interest
- compare them to a standard set of rules
- produce reports detailing both normal and abnormal results.

Electrospray Ionization

Electrospray ionization is a soft ionization technique that allows for the mass spectrometric analysis of labile, polar compounds that were previously extremely difficult to analyze. A soft ionization technique is one in which the majority of the ionic species observed are attributable to molecular related species or facile fragmentation (e.g. dehydration) from the parent molecule. A solution of the analyte of interest is nebulized from a fine nozzle through a high electric field, forming the ions to be analyzed.

In a typical experiment the mobile phase pumped from an LC system, or an infusion pump, enters through the probe and is pneumatically converted to an electrostatically charged aerosol spray. The solvent is evaporated from the spray by means of a drying gas passed through the desolvation heater. The resulting analyte and solvent ions are drawn through the sample cone aperture into the ion block, from where they are extracted into the analyzer.

The electrospray ionization technique allows rapid, accurate and sensitive analysis of a wide range of analytes from low molecular weight polar compounds (less than 200 Da) to biopolymers larger than 100 kDa. Generally, compounds of less than 1000 Da produce singly charged protonated molecules ($[M+H]^+$) in positive ion mode. Likewise, these low molecular weight analytes yield deprotonated molecules ($[M-H]^-$) ions in negative ion mode, although this is dependent upon compound structure.

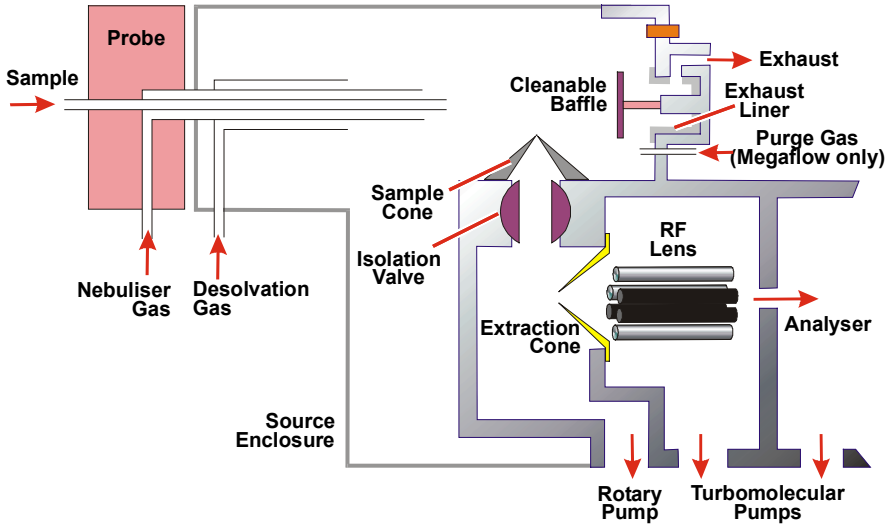


Figure 1.1 The Z-spray ionization source

High mass biopolymers, for example peptides, proteins and oligonucleotides, produce a series of multiply charged ions. The acquired data can be transformed by the software to give a molecular weight profile of the biopolymer.

The Z-spray source can be tuned to fragment ions within the 'ion block', although the 'cone voltage' is usually tuned for optimum sensitivity. The optimum cone voltage setting is normally compound-class dependent. Cone voltage fragmentation can provide valuable structural information of low molecular weight analytes, although it is considerable less specific than the experiments detailed below.

Electrospray is a concentration dependant technique, and it is this characteristic that is taken advantage of for the quantification process. Analytes and the isotopically labeled analogs both have a similar sensitivity under ionization, and the relative peak intensities detected will be proportional to their concentration in the solution under analysis.

Analytical Procedures

The following analyses have been developed and reported in the literature, and are in use in one form or another in a number of laboratories around the world. They are explained in more detail in the references cited at the end of this document.

Analysis Of Acylcarnitines

L-carnitine (3-hydroxy-4-aminobutyrobetaine) plays a vital role in the mitochondrial β-oxidation of fatty acids by acting as a transporter of acyl groups across mitochondrial membranes. Free fatty acids are activated by the cytosol to the coenzyme A thioesters (acyl CoA) and conjugated with carnitine by long-chain carnitine acyltransferase. This is also known as carnitine palmitoyl transferase and is located on the inner and outer mitochondrial membranes. When the carnitines cross the membrane, the carnitine is regenerated with an equivalent release of the acyl-CoA. Successive cycles of β-oxidation are carried out by a closely coupled system of enzyme complexes, of which the acyl-CoA dehydrogenases are essential components. Three specific systems exist with overlapping chain-length specificity, very long-chain (VLCAD), medium-chain (MCAD) and short-chain (SCAD), whose preferred substrates are palmitoyl (C_{16}) CoA, octanoyl (C_8) CoA and butyryl (C_4) CoA, respectively. One of the major products of the β-oxidation, acetyl (C_2) CoA, is transported out of the mitochondrion as a carnitine conjugate.

Defects have been identified and characterized in many of these enzymes and, in disorder of fatty acid and branched chain amino acid catabolism affecting the mitochondrial enzymes, the potential exists for the accumulation of abnormal acyl-CoA intermediates. These compounds may exhibit toxicity by inhibiting other essential enzymes and may also sequester the limited pool of coenzyme A in the mitochondrion. Carnitine plays an important role in the transport of these intermediates out of the mitochondrion using the carnitine acyltransferases. The ratio of bound carnitine (acylcarnitine) to free carnitine in plasma reflects the acyl CoA to free CoA thioester ratio, and the ratio is usually elevated in-patients with fatty acid oxidation disorders. The secondary role of carnitine is therefore one of detoxification when there is an abnormal accumulation of abnormal acyl CoA.

The generation of a profile of the acylcarnitines present in the blood should yield a significant amount of information, relating to the fatty acid catabolism of the patient.

Acylcarnitines can be analyzed in the native state but exist in solution as zwitter ions. If a positive ion analysis is to be performed considerable increases in sensitivity are achieved by blocking the carboxylate group through esterification. If free acyl-carnitines are to be analyzed, the addition of a small amount of acid will serve to promote the fraction of the positive ions in solution. The discussions in this document relate primarily to the analysis of butyl esters.

The acylcarnitines may be studied by an analysis where all the molecular species that decompose to yield a common fragment ion are determined. In mass spectrometric terms, this is called a PRECURSOR or PARENT ion scan. In the analysis of acylcarnitines, a common fragment of mass 85 Da can be used to provide information on all the butylated acylcarnitines present in the sample. The derivatization and fragmentation is shown in Figure 1.2.

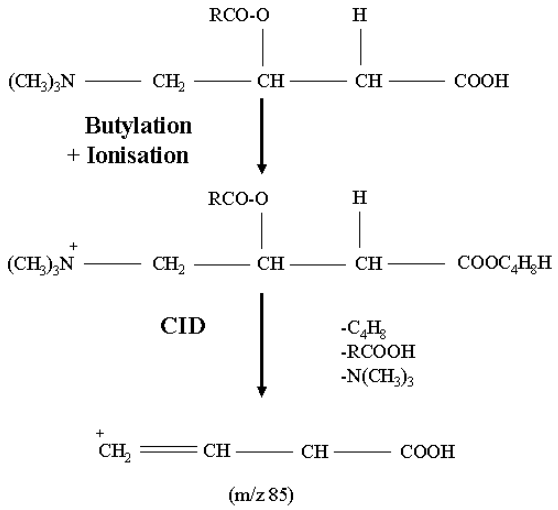


Figure 1.2 Butylation and fragmentation of acylcarnitines to yield a common fragment at m/z 85

The acylcarnitine profile is derived by scanning the first mass analyzer (MS1), and only allowing fragment ions of *m/z* 85 to pass through the second mass analyzer (MS2) to be detected. The PARENT or PRECURSOR ion scan is depicted in Figure 1.3.

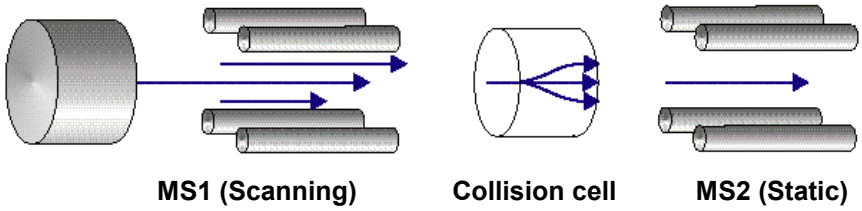


Figure 1.3 A schematic representation of the parent or precursor ion scan in which the first mass analyzer (MS1) is scanned and the second mass analyzer (MS2) is set to pass only one specific fragment

A typical acylcarnitine profile from a healthy patient is shown in Figure 1.4. The spectrum shows the presence of free carnitine (*m/z* 218), acetyl carnitine (*m/z* 260), propionyl carnitine (*m/z* 274) and palmitoyl carnitine (*m/z* 456). Four internal standards are used for quantification purposes (tri-deuterated free carnitine, propionyl carnitine, octanoyl carnitine and palmitoyl carnitine).

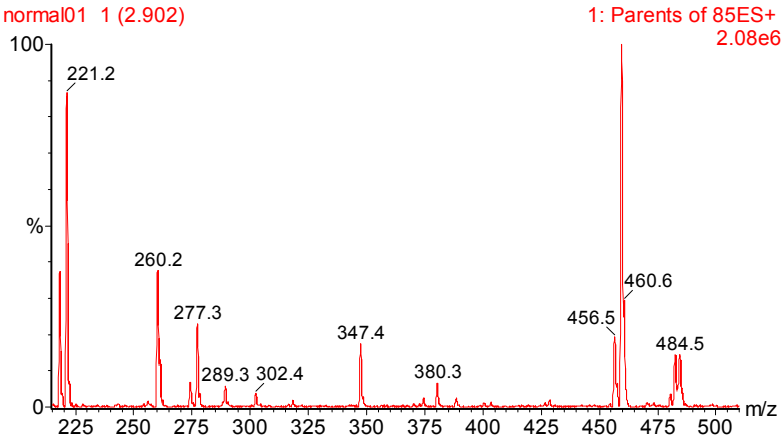


Figure 1.4 Acylcarnitine profile from a healthy patient

Table 1.1 shows a list of the masses that may be observed for a number of acylcarnitine butyl esters.

Notation	Common Name	Mass of butyl ester /Da
Free	Free carnitine	218.18
d_3-Free	d_3-free carnitine (internal standard)	221.20
C_2	Acetyl	260.19
d_3-C_2	d_3-acetyl (internal standard)	263.21
C_3	Propionyl	274.20
d_3-C_3	d_3-propionyl (internal standard)	277.22
C_4	Butyryl	288.22
d_3-C_4	d_3-butyryl (internal standard)	291.24
$C_{5:1}$	Tiglyl	300.22
C_5	Isovaleryl	302.23
C_6	Hexanoyl	316.25
C_5-OH	3-hydroxy-isovaleryl	318.23
$C_{8:1}$	Octenoyl	342.27
C_8	Octanoyl	344.28
d_3-C_8	d_3-octanoyl (internal standard)	347.30
$C_{10:2}$	Decadienoyl	368.28
$C_{10:1}$	Decenoyl	370.30
C_{10}	Decanoyl	372.31
C_4-DC	Methylmalonoyl	374.29
C_5-DC	Glutaryl	388.31
$C_{12:1}$	Dodecenoyl	398.33
C_{12}	Dodecanoyl	400.34
C_6-DC	Adipyl	402.33
$C_{14:2}$		424.34
$C_{14:1}$		426.36
C_{14}	Myristoyl	428.37
C_8-DC	Subaryl	430.41
C_{14}-OH	3-hydroxy-myristoyl	444.37
$C_{16:1}$	Palmitoleyl	454.39
C_{16}	Palmitoyl	456.40
C_{10}-DC	Sebacyl	458.45
d_3-C_{16}	d_3-palmitoyl (internal standard)	459.42
$C_{16:1}$-OH	3-hydroxy palmitoleyl	470.40
C_{16}-OH	3-hydroxy palmitoyl	472.40
$C_{18:2}$	Linoleyl	480.40
$C_{18:1}$	Oleyl	482.42
C_{18}	Stearoyl	484.44
$C_{18:2}$-OH	3-hydroxy linoleyl	496.40
$C_{18:1}$-OH	3-hydroxy oleyl	498.42

Table 1.1 Masses of butyl esters

Analysis Of Amino Acids

A number of inherited diseases result in the disruption of the metabolism and transformation of amino acids. This results in an accumulation of certain amino acids, depending on the reduction in enzyme efficiency that has occurred.

In phenylketonuria, a deficiency in phenylalanine hydroxylase inhibits the conversion of phenylalanine to tyrosine, with an accumulation of phenylalanine and a decrease in tyrosine.

An elevation of methionine in blood may be indicative of two inherited amino acid metabolic disorders: isolated hypermethioninemia and homocystinuria due to cystathionine β-synthase deficiency.

Maple syrup urine disease is an inherited disorder characterized by the accumulation of the branched-chain amino acids and their corresponding α-keto acids in blood and urine. It arises from defects in the oxidative decarboxylation by branched-chain α-keto-acid dehydrogenase (a multienzyme complex).

Early detection and dietary treatment of these and other disorders can lead to a significantly improved outcome for a patient.

A large number of amino acids (though not all) may be analyzed using tandem mass spectrometry. The metabolites are extracted from the blood spots using methanol and derivatized using the same procedure as for the acylcarnitines. The fragmentation of the esterified amino acids is somewhat different to the acylcarnitine, and amino acids fall into two distinct classes for their detection by tandem mass spectrometry, based on structural and chemical differences. In addition, both glycine and arginine show significantly improved sensitivity if different scanning methods are used.

Neutral and Acidic Amino Acids

In the case of the derivatized amino acids it is possible for all the amino acids to lose a neutral molecule of mass 102 Da. In mass spectrometric terms, detection of all molecular species that undergo a CONSTANT NEUTRAL LOSS of 102 Da will provide information on the different butylated amino acids present in the sample.

The fragmentation for this transition is shown in Figure 1.5.

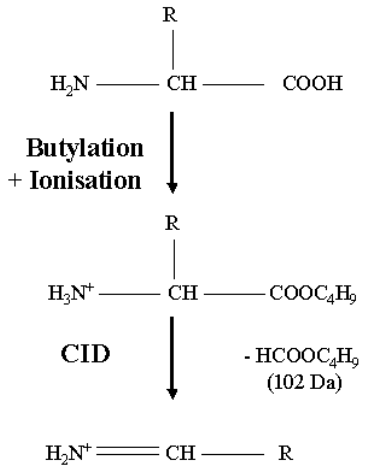


Figure 1.5 Butylation and fragmentation of amino acids to show the common loss of butyl formate (102 Da)

The neutral and acidic amino acid profile is derived by scanning the first and second mass analyzers together with the difference between the first mass analyzer (MS1) and the second mass analyzer (MS2) set to the neutral loss mass, 102 Da. The only time an ion will be detected is when the fragmentation of the species in the collision cell results in the appropriate loss from the precursor ion. The CONSTANT NEUTRAL LOSS scan is depicted in Figure 1.6.

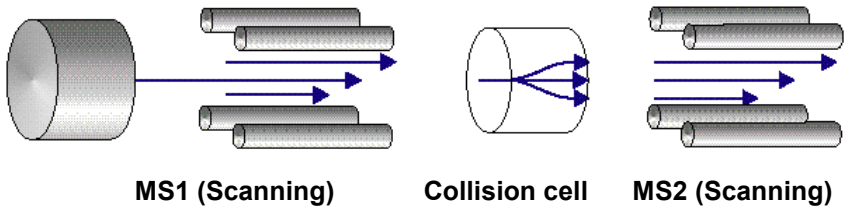


Figure 1.6 A schematic representation of the constant neutral loss scan in which both mass analysers (MS1 and MS2) are scanned at a pre-defined offset of 102 Da for the detection of neutral amino acids

A neutral amino acid spectrum from a healthy patient is shown in Figure 1.7.

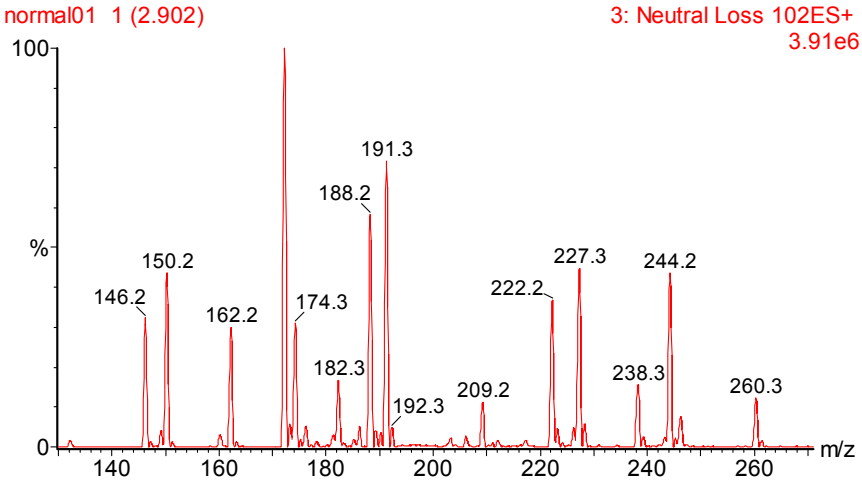


Figure 1.7 Neutral amino acid profile from a healthy patient

The amino acids detected in this spectrum are detailed in Table 1.1. In addition to the endogenous species detected, a number of deuterated internal standard analogues of the amino acids have also been included for quantification purposes. The internal standards used in the experiment shown in Figure 1.7 include: d_4-alanine (*m/z* 150), d_8-valine (*m/z* 182), d_3-leucine (*m/z* 191), d_3-methionine (*m/z* 209), d_5-phenylalanine (*m/z* 227) and d_6-tyrosine (*m/z* 244).

Basic Amino Acids

In instances where the side chain of the amino acid contains a basic group, it is energetically more favorable for the derivatized compounds to lose an ammonia molecule (an additional 17 Da) as well as the butyl formate molecule. Again, the loss from the precursor molecule will be neutral (losing two species instead of one), but in such cases all ions undergoing a CONSTANT NEUTRAL LOSS of 119 Da will need to be reported.

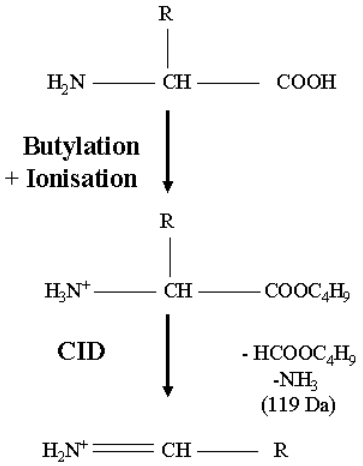


Figure 1.8 Butylation and fragmentation of amino acids to show the common loss of butyl formate and ammonia (119 Da)

The basic amino acid profile is derived by scanning the first and second mass analyzers together with the first mass analyzer (MS1) set to the neutral loss mass, 119 Da. The only time an ion will be detected is when the fragmentation of the species in the collision cell results in the appropriate loss from the precursor ion. The CONSTANT NEUTRAL LOSS scan is depicted in Figure 1.9.

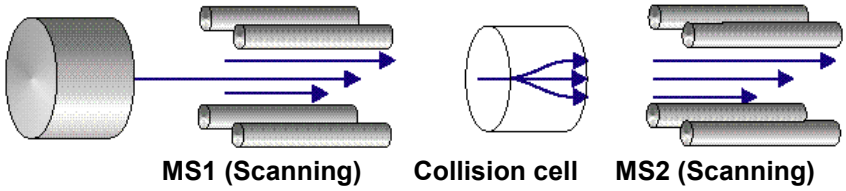


Figure 1.9 A schematic representation of the constant neutral loss scan in which both mass analysers (MS1 and MS2) are scanned at a pre-defined offset of 119 Da for the detection of basic amino acids

A basic amino acid spectrum from a healthy patient is shown in Figure 1.10.

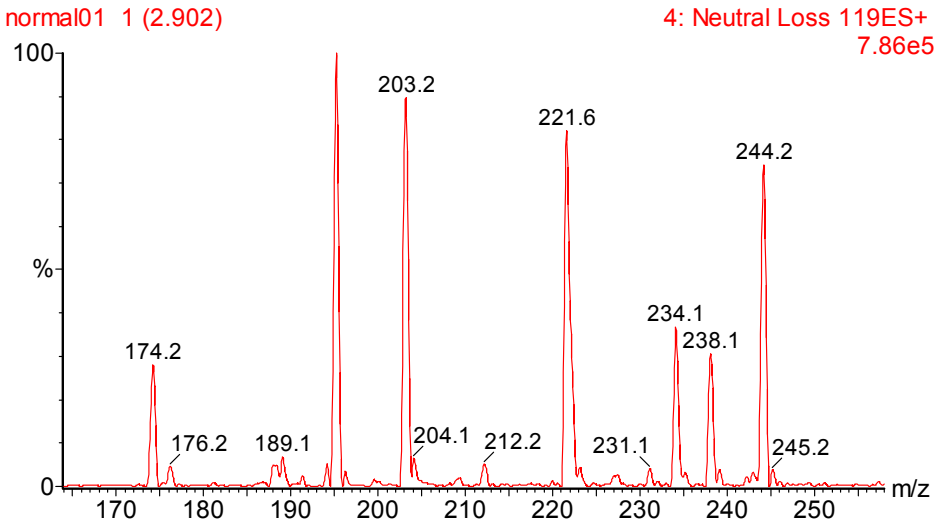


Figure 1.10 Basic amino acid profile from a healthy patient

The amino acids detected in this spectrum are detailed in **Table 1.2**. In addition to the endogenous species detected, a single deuterated internal standard, d_6-ornithine (*m/z* 195) has been included for quantification purposes.

Note: some amino acids appear in both the 102Da and 119Da Neutral loss spectra.

Glycine and Arginine

Two exceptions to the general classifications for amino acids described above relate to glycine and arginine.

Although glycine can be detected by using a constant neutral loss of 102 Da, examination of the fragmentation of the glycine butyl ester using a daughter ion scan shows that the most abundant fragment results from the neutral loss of C_4H_8 (56 Da).

A DAUGHTER or PRODUCT ion scan may be generated by passing the analyte ion of interest through the first mass analyzer (MS1), fragmenting the selected ion by collision with a target gas in the collision cell, and scanning the second mass analyzer (MS2) in order to detect all the fragments generated from the selected precursor. This is illustrated in Figure 1.11.

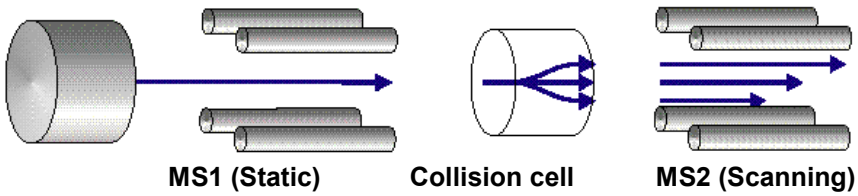


Figure 1.11 A schematic representation of the daughter or product ion scan in which the first mass analyzer (MS1) is fixed to pass the ion of interest and the second mass analyzer (MS2) is scanned to detect all the fragment ions generated in the collision cell.

The product ion spectrum from the glycine butyl ester is shown in Figure 1.12.

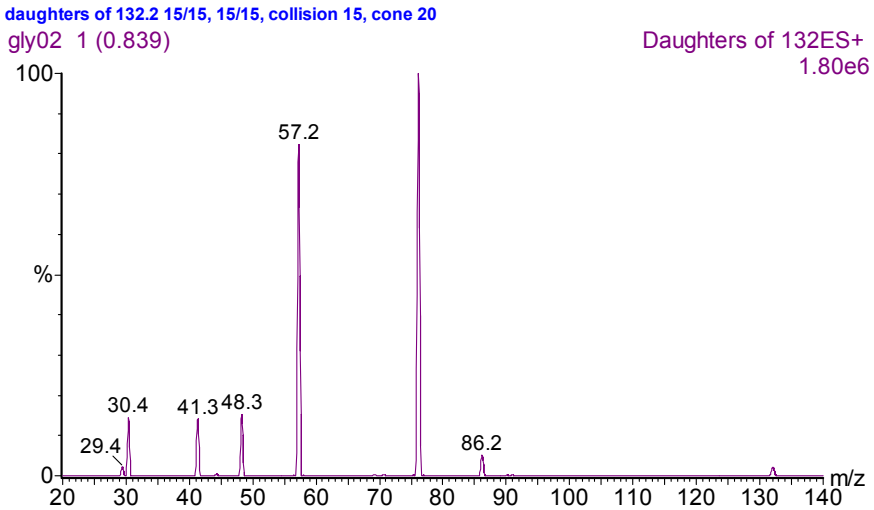


Figure 1.12 Product ion spectrum of the butyl ester of glycine. Cone voltage = 20V, Collision energy = 15eV

Amino Acid	Sigma #	Mass of ester +H	Preferred loss
Alanine	A7503	146.12	102.07
Arginine	A4881	231.18	161.11
Asparagine	A8256	189.12	119.09
Aspartic acid	A9006	246.18 (2 Bu)	102.07
Citrulline	C6131	232.17	119.09
Glutamic acid	G1126	260.19 (2 Bu)	102.07
Glycine	G7126	132.10	56.06
Histidine	H7875	212.14	102.07
Isoleucine	I5393	188.16	102.07
Leucine	L7875	188.16	102.07
Lysine	L6001	203.18	119.09
Methionine	M9500	206.12	102.07
1-Me-Histidine	M9005	226.16	102.07
3-Me-Histidine	M3879	226.16	102.07
Norleucine	N1398	188.16	102.07
Ornithine	O2250	189.16	119.09
Phenylalanine	P1876	222.15	102.07
Proline	P0255	172.13	102.07
Serine	S4375	162.11	102.07
Threonine	T8375	176.13	102.07
Tryptophan	T0129	261.16	102.07
Tyrosine	T3379	238.14	102.07
Valine	V0375	174.15	102.07

Table 1.2 List of common amino acids and preferred neutral loss.

The product ion spectrum of the glycine butyl ester shows the protonated molecule $(M+H)^+$ at *m/z* 132. As the fragment ion at *m/z* 76 (loss of 56 Da) is approximately five times more abundant than the normal neutral loss of 102Da, it is more appropriate to analyse for glycine using a neutral loss of 56Da.

A neutral loss of 56Da is also observed for the other butyl esters of the amino acids, but is not the most intense transition in the product ion spectra.

Arginine is another anomally from the normal fragmentations described above. Although arginine is observed in the basic amino acid profile (neutral loss of 119 Da), the most abundant transition is the loss of 161Da from the *m/z* 231 precursor to give a dominant product ion at *m/z* 70.

A list of the common amino acids, their masses, and preferred neutral loss, is in **Table 1.2**.

Bile Acids

Bile acid concentrations are elevated in the blood of some neonates with cholestatic hepatobiliary disorders, providing a means of screening for treatable conditions including biliary atresia. Bile acids can be eluted from the Guthrie blood spots using methanol containing deuterated internal standards, the glycine and taurine conjugates of d_4-chenodeoxycholic acid and d_4-cholic acid. The samples are evaporated to dryness and reconstituted in propan-2-ol/water (60/40 *v/v*) and analyzed directly by negative ion electrospray tandem mass spectrometry.

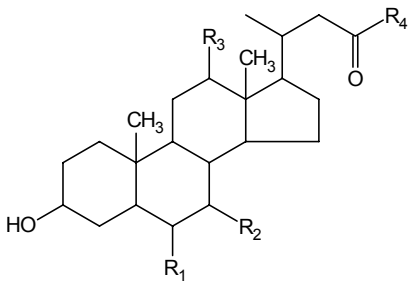


Figure 1.13 Generic Structure of Bile Acids

Name	R_1	R_2	R_3	R_4	$(M-H)^-$
Lithocholic	H	H	H	OH	375.29
Chenodeoxycholic	H	OH	H	OH	391.28
Deoxycholic	H	H	OH	OH	391.28
Hyodeoxycholic	OH	H	H	OH	391.28
Cholic	H	OH	OH	OH	407.28
Hyocholic	OH	OH	H	OH	407.28
Glycolithocholic	H	H	H	$NH-CH_2-COOH$	431.31
Glycodeoxycholic	H	H	OH	$NH-CH_2-COOH$	448.31
Glycocholic	H	OH	OH	$NH-CH_2-COOH$	464.30
Glycochenodeoxycholic	H	OH	H	$NH-CH_2-COOH$	448.31
Taurochenodeoxycholic	H	OH	H	$NH-(CH_2)_2-SO_3H$	498.29
Taurodeoxycholic	H	H	OH	$NH-(CH_2)_2-SO_3H$	498.29
Taurolithocholic	H	H	H	$NH-(CH_2)_2-SO_3H$	482.29
Taurocholic	H	OH	OH	$NH-(CH_2)_2-SO_3H$	514.28

Table 1.3 Structure of generic bile acids

Bile acids form strong deprotonated molecules, giving rise to a related molecular species at the *m/z* value shown in Table 1.3. Predominant losses from the deprotonated molecules are the consecutive losses of water (H_2O), depending on the number of substituent hydroxyl groups. Neutral losses of 36 Da and 54 Da may be used for the determination of di- and tri- hydroxy bile acids in, for example a LC-MS separation experiment.

The majority of work analyzing bile acids from neonatal blood spots has been performed using the common fragmentation of the glycine and taurine conjugated bile acids. Taurine conjugated bile acids show a common fragment (relating to the taurine sub-unit) of *m/z* 80, formed from the deprotonated molecule. The glycine conjugated bile acids show a common fragment (relating to the glycine sub-unit) of *m/z* 74, formed from the deprotonated molecule.

Owing to the small number of species to be analyzed (it is not possible to differentiate the different bile acid isomers in a simple MS/MS experiment), it is usual to use multiple reaction monitoring to monitor specific species (and the internal standards) for quantification purposes. The limit of detection of the conjugated bile acids extracted from 5mm blood spots is less than 0.5μM for each isobaric species.

Inclusion of the bile acid screening into a common experiment with amino acids and acylcarnitines has been undertaken on a limited basis. One problem that occurs during the derivatization (butylation) process is the hydrolysis of the glycine groups from the conjugates. The taurine conjugates are not affected.

Other Analyses

A considerable amount of research has been devoted to the analysis of hemoglobin by mass spectrometry and tandem mass spectrometry. It is possible to use single stage mass spectrometry to measure the level of total hemoglobin glycation by using a simple dilution of whole blood. The

same sample may also be used in the preliminary stages of the detection and characterization of hemoglobin variants. The experimental procedures involved in such characterizations fall outside the scope of this document, but are available on request.

Modes Of Data Acquisition

If a number of different classes of metabolites are to be screened for, the mass spectral data may be acquired in one of two ways:

1. A method where a **mass spectrum** is acquired in order to detect all the species that undergo a specific decomposition (e.g. PARENTS of *m/z* 85 will yield a spectrum to show all masses of all the butylated acylcarnitines present in the sample). This method will give rise to a spectrum that may indicate a known disorder, or may give rise to a signal that has not previously been observed. This method will be referred to as the **full-scan MCA** mode of operation.

2. A method where only specific fragmentations are detected by the mass spectrometer using a multiple reaction monitoring technique (MRM). In this mode of operation only specific user-defined metabolites will be detected and all other metabolites will be ignored. This method will be referred to as the **MRM** mode of operation.

The choice of scanning method depends on a number of factors, including legislative and ethical considerations. It might be considered that the MRM mode of operation would be preferred for a neonatal screening application as it is usual to just test for a specific disorder. MRM data files are considerably smaller than MCA data files acquired for similar compound classes. However, in cases where samples from acute patients are to be studied it will be necessary to examine the spectra for all possible disorders, and the full-scan MCA method will be required. These different modes of scanning the mass spectrometer to derive information are termed FUNCTIONS.

Despite the complex physiology involved, the levels of the acylcarnitine and amino acid metabolites present in blood are reasonably constant from person to person. If a metabolic disorder is apparent then the levels of the appropriate metabolite are altered severely, usually increased, by a measurable difference from what is considered to be 'normal'.

In order to develop a consistency in the measurements, known amounts of isotopically labeled acylcarnitines and amino acids are added to the blood sample while it is being extracted from the filter paper. The internal standards, which are identical in every way to the endogenous metabolites apart from the isotopic labeling, behave chemically in exactly the same way as the metabolites they are mimicking. This means that they will also be detected by the different scan functions of the mass spectrometer. The relative levels of the endogenous metabolites to the internal standards can easily be determined by measuring the relative intensities of the peaks in the acquired mass spectra. It is not economically viable to provide an internal standard for every analyte that may appear in a physiological sample, so only a limited number of internal standards are used. The range of internal standard materials available is discussed in more detail in the next section.

Chapter 2 Sample Preparation and Data Acquisition

Table of Contents

Figures

Sample Preparation

Blood samples are typically collected on Guthrie filter cards (Schleicher and Schuell 903 filter paper), dried and submitted to central laboratories for analysis. These cards have been in use since the mid 1960s and remain in routine use around the world. Blood spots are punched from these filter cards and used in a variety of ways. A number of different sizes of blood spots are typically used, corresponding to different amounts of whole blood equivalent, and these are summarized in the table below.

Spot diameter /mm	Area / mm^2	Whole blood content / µL
3	7.1	3.1
4.7	17.3	7.6
6	28.3	12.4
2 x 4.7	34.7	15.2

Table 2.1 Blood spot diameter showing whole blood equivalent

Preparation of samples for introduction into the mass spectrometer is perhaps the most time-consuming part of the whole analysis. The sample preparation procedure described here is that derived by the group at Duke University Medical Centre (D S Millington, D L Norwood, N Kodo, C R Roe and F Inoue, *Anal. Biochem.*, **180** (1989) 331-339 and D H Chace, D S Millington, N Terada, S G Kahler, C R Roe and L F Hoffman, *Clin. Chem.*, **39** (1993) 66-71) and is based on the analysis of blood extracted from Guthrie cards

Reagents

The reagents used for the derivatization are;

Reagent	Grade	Use
Methanol	HPLC grade	for extraction
Butanolic HCl	see below	for derivatization
Acetonitrile	HPLC grade	for reconstituting the derivatized samples
Water	HPLC grade	for reconstituting the derivatized samples

Butanolic Hydrochloric acid
The derivatizing reagent is probably the most critical of the reagents used. The derivatization requires the presence of acid in order to catalyze the reaction. The concentration of the hydrochloric acid is normally stated as 3M. However, the reagent is required to be anhydrous, as the presence of water during the derivatization stage will result in hydrolysis of the acylcarnitines, and to some extent, the amino acids, thus reducing the sensitivity of the overall analysis. Butanolic HCl is commercially available in some countries, but there are limitations on shipping, making access to such sources troublesome.

Butanolic HCl may be prepared in one of the following ways:

- Adding acetyl chloride to dry n-butanol in the ratio 1:9. This is an exothermic process, and due care must be exercised during the mixing. It is usual to prepare the reagent with the mixing vessel in an ice bath.

- Passing hydrochloric acid gas through dry n-butanol. The gas may be sourced from, either a cylinder or from the reaction of sodium chloride with sulfuric acid. Passing the gas for 45 minutes should result in an appropriate acid concentration in the solution. The concentration of the acid may be verified by back titration.

Extraction and Derivatization Procedure

Extraction and derivatization of the metabolites should be performed in appropriate vessels. Glass vials have been used successfully, but the requirement for analyzing large numbers of samples makes the use of single vials for each sample somewhat unwieldy. Preference is currently being given to the use of 96-well microtitre plates. The plate material and well-depth should be chosen with care as some materials have been shown to degrade with the reagents used. Polypropylene plates have been shown to be particularly resistant to degradation. The profile of the wells is not critical, but round-bottomed vials will result in a concentration of the analytes of interest in the bottom of the wells during the drying procedures.

The blood spot samples are punched into the chosen vessels and a volume of methanol is added to effect the selective extraction of the metabolites from the filter paper. In this process the blood proteins remain attached to the filter paper. The samples are then gently agitated for approximately 30 minutes, although there have been reports of efficient extraction by simply allowing the samples to stand.

The methanol also contains the isotopically labeled internal standards that will subsequently be used for quantification purposes. Isotopically labeled standards are used as they will have similar chemical and ionization characteristics to the analytes of interest, but will differ in their molecular masses, and will be differentiated during the analysis by the tandem mass spectrometer. Adding the internal standards at the point of sample extraction also serves as a check that the derivatization has been effective. If Internal Standard peaks are not at the expected intensity then the derivatization may not have worked.

The choice of which internal standards to add will depend on the particular analytes of interest. In an ideal situation, every analyte to be measured would require the addition of an appropriately labeled chemical analogue, although this may not be cost effective. The concentration of the internal standards in the methanol will depend on:

(i) The volume of blood (number and size of blood spot punches) used.

(ii) The volume of methanol used for the extraction.

(iii) The clinical decision point for the concentration of a particular analyte.

This final point is regiospecific and it is recommended that the internal standards are added at a concentration equivalent to the clinical concentration that prompts follow up (e.g. approximately 200μM/L equivalent for d_5-phenylalanine for measuring phenylalanine in whole blood). The highest accuracy of measurement will be achieved when the intensity of the analyte and internal standard peaks are approximately the same intensity.

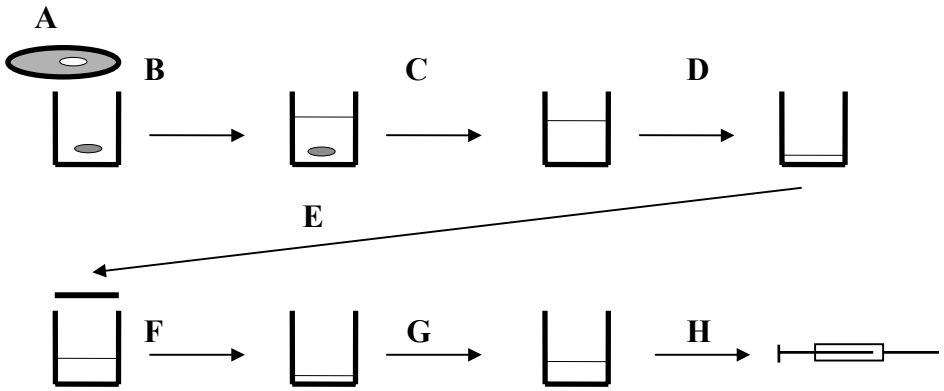
Once the metabolites have been extracted, it is necessary to either transfer the methanolic solution to another vessel, or remove the blood spot. The methanol is then evaporated under a stream of air or nitrogen (possibly with gentle heating to aid the evaporation), to leave a residue of the extracted metabolites in the bottom of the well. The evaporation time will depend on a number of factors and may take up to 20 minutes.

An amount of butanolic HCl (60-100μL) is then added to each dried sample. The sample vessels then need to be sealed to prevent evaporation of the reagents before the derivatization is complete. A number of commercial, inert rubber plates are available for this purpose. The sealed plate is then heated at 60°C for approximately 15 minutes. The optimum conditions for butylation should be carefully established. Some evidence exists for the hydrolysis of acylcarnitines if a prolonged butylation period is used.

Following the derivatization, it is necessary to remove the butanolic HCl by evaporation, similar to the process described above. Hydrochloric acid vapors are both toxic and corrosive, and care must be exercised. As the viscosity of n-butanol is somewhat higher than methanol, this stage is usually the most time-consuming of the whole procedure.

Once the derivatized samples have been dried down, they are reconstituted in an appropriate solvent (e.g. 80% acetonitrile) in preparation for injection into the mass spectrometer.

The derivatization procedure described above is shown schematically Figure 2.1.



A. Punch out selected spot from Guthrie card.

B. Add MeOH (e.g 200μL, including internal standard) and agitate for 20 minutes.

C. Decant off supernatant or remove bloodspot.

D. Evaporate to dryness under nitrogen or air with mild (40°C) heating.

E. Add 60-100μL butanolic HCl. Cap or seal. Heat at 60°C for 15 minutes.

F. Evaporate to dryness under nitrogen or air with mild (40°C) heating.

G. Add suitable solvent (approx. 50-100μL) e.g. 80% aqueous MeCN

H. Inject appropriate amount (typically 10-20μL) into mobile phase flowing to the mass spectrometer.

Figure 2.1 Schematic diagram of sample preparation

It is possible that the operations in the derivatization process may be automated such that batches of samples are prepared in a single session.

Isotopically Labeled Internal Standards

The primary source of isotopically labeled internal standards is:

Cambridge Isotope Laboratories
50 Frontage Road
Andover, MA 01810-5413
USA

Phone: 508-749-8000
FAX: 508-749-2768

Please check for a local supplier. The UK catalogue numbers are shown below (Correct at Spring 2001).

Compound	CIL Catalogue #
$^{2}H_{4}$- alanine	DLM-1276
$^{2}H_{3}$-aspartic acid	DLM-832
$^{2}H_{3}$-glutamic acid	DLM-335
$^{2}H_{5}$-glycine	DLM-280
$^{13}C^{15}N$-glycine	CNLM-507
$^{2}H_{3}$-leucine	DLM-1259
$^{2}H_{3}$-methionine	DLM-431
$^{2}H_{5}$-phenylalanine	DLM-2986
$^{2}H_{7}$-proline	DLM-487
$^{2}H_{2}$-serine	DLM-1073
$^{2}H_{2}$-tyrosine	DLM-449
$^{2}H_{4}$-tyrosine	DLM-451
$^{2}H_{8}$-valine	DLM-311
==========	==========
$^{2}H_{3}$-carnitine	DLM-1871
$^{2}H_{6}$-carnitine	DLM-3820
$^{2}H_{9}$-carnitine	DLM-3555
$^{2}H_{3}$-C2-carnitine	DLM-754
$^{2}H_{6}$-C2-carnitine	DLM-3821
$^{2}H_{9}$-C2-carnitine	DLM-3822
$^{2}H_{3}$-C3-carnitine	DLM-3973
$^{2}H_{3}$-C4-carnitine	DLM-3861
$^{2}H_{9}$-C5-carnitine	DLM-3974
$^{2}H_{3}$-C8-carnitine	DLM-755
$^{2}H_{3}$-C16-carnitine	DLM-1263

A primary source of kits of isotopically labelled internal standards is:

NeoGen Screening Inc.
110 Roesslar Road
Pittsburgh, PA 15220, USA
Phone: 412-341-8658
FAX: 412-341-8926

NeoGen Screening supply two kits that contain certified solutions of L-amino acids (Kit A) and L-carnitine and acylcarnitines (Kit B). Each kit should provide sufficient internal standards to allow for the preparation of up to 20,000 3mm blood spots.

Kit A	Kit B
$^{15}N^{13}C$-glycine	$^{2}H_9$-L-carnitine
$^{2}H_4$-alanine	$^{2}H_3$-acetylcarnitine
$^{2}H_8$-valine	$^{2}H_3$-propionylcarnitine
$^{2}H_3$-leucine	$^{2}H_3$-butyrylcarnitine
$^{2}H_3$-methionine	$^{2}H_9$-isovalerylcarnitine
$^{2}H_5$-phenylalanine	$^{2}H_3$-octanoylcarnitine
$^{2}H_4$-tyrosine	$^{2}H_9$-myristoylcarnitine
$^{2}H_3$-aspartic acid	$^{2}H_3$-palmitoylcarnitine
$^{2}H_3$-glutamic acid	
$^{2}H_2$-ornithine	
$^{2}H_2$-citrulline	
$^{2}H_4{}^{13}C$-arginine	

It is advisable to maintain close links with the suppliers, as it is understood that new labeled compounds that might be suitable for this type of work are constantly under development.

Some isotopically labeled acylcarnitines are also available from:

Dr Herman ten Brink
Department of Pediatrics
Academic Hospital V U Metabolic Unit
P O Box 7057
1007 Amsterdam
Netherlands

Phone: + 31-20-444-2883
FAX: + 31-20-444-0305

Sample Introduction

Once the sample has been prepared and is in solution, it needs to be passed into the ion source of the mass spectrometer. This is normally performed by injecting the sample into an appropriate mobile phase, which is flowing continuously into the electrospray ion source. There are no special restrictions on the type of LC pump and autosampler that may be used for the NeoLynx application.

The mobile phase is usually delivered to the mass spectrometer by an LC pump at flow rates from 10µL/min to 100µL/min, depending on the application. A typical mobile phase for the majority of applications is 50-80% aqueous acetonitrile or aqueous methanol containing no additives. The lack of additives allows for simple switching between positive and negative ion analyses with minimal loss in sensitivity. A number of LC pumps may be directly controlled from MassLynx, allowing user-defined flow rates to be programmed.

In order to introduce the sample into the mobile phase some form of sample injection is necessary. This may be performed using a manual LC injector (e.g. Rheodyne 7125), but is more commonly performed using an LC autosampler. Autosamplers allow large batches of samples to be injected without user intervention. A range of LC autosamplers may be controlled *via* MassLynx, and used for synchronizing the sample injection with a 'start' signal to the mass spectrometer

The facility is also available for the inclusion of an ultra-violet (UV) or diode array (DAD) detector to be controlled from the data system, if required (this will only be required for adding an extra dimension of information when performing chromatographic separations).

For details of how to configure the pump, autosampler and detector see the Controlling Inlet Systems and Autosamplers chapter in the MassLynx Guide to Data Acquisition.

Mass Calibration

Periodic verification of the mass scales of both analyzers is recommended for optimum performance in both full-scan and MRM acquisitions.

It is advised that the mass scales should be calibrated to either *m/z* 23 (sodium) using a mixture of sodium iodide and rubidium iodide (nairb.ref). The upper limit of the mass calibration should be greater than *m/z* 600.

For full details of calibration procedures and recipes for calibration solutions see the Mass Calibration section in the Quattro LC User's Guide.

Mass Spectrometer Tuning

In order to get the best analysis from the mass spectrometer, it is important that the instrument is tuned effectively for the analysis. It is recommended that a 'standard' normal blood spot is prepared with each batch, and that this sample is used for tuning the mass spectrometer. Ideally, a copy of the tune page should be printed and stored for future reference and quality control.

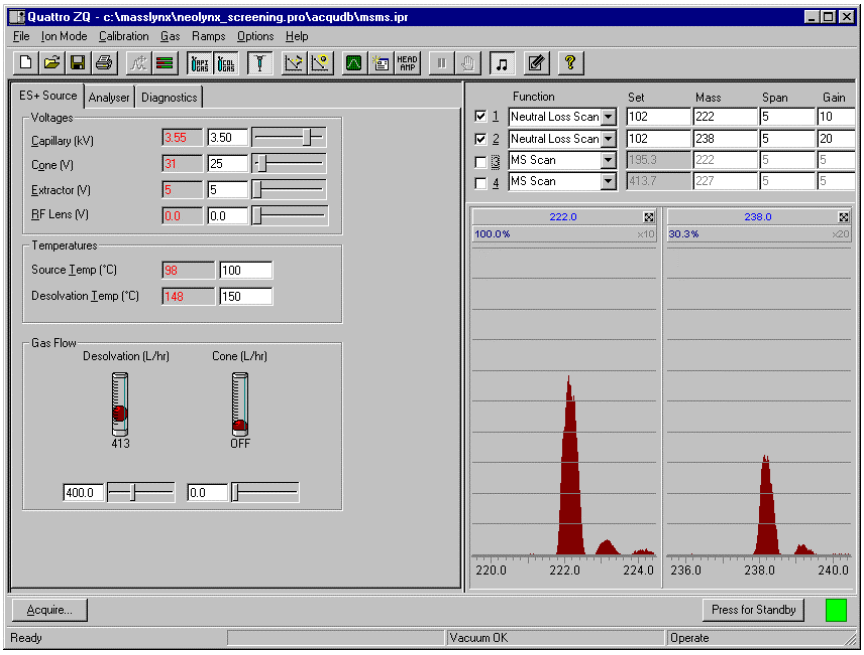


Figure 2.2 Typical tune page for Quattro *Micro* Z-spray

When tuning the instrument, it is possible to monitor up to four peaks on the tune page. A typical tune page setting for neonatal blood spot analysis on amino acids and acylcarnitines is shown in Figure 2.2.

To access the tune page select the **Instrument ,Tune Page** from the MassLynx Shortcut Bar. The collision gas pressure for argon (recommended) is approximately 2.0 x 10^{-3} mbar (indicated on the readbacks).

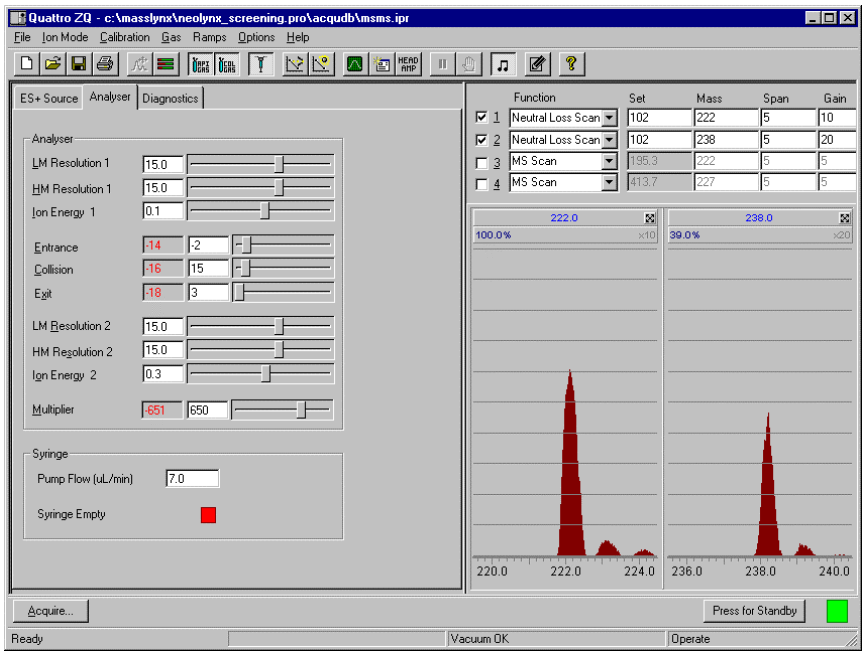


Figure 2.3 Typical tune page for Quattro *Micro* Z-spray

The *italicized* parameters are intended as a guide only and must be set according to the resolution required.

Source

Capillary	3.50 kV
Cone	set in scan function editor
Extractor	3-5V
RF Lens	0.0V
Block temperature	140ºC
Desolvation Temperature	300°C

Analyzer

LM resolution1	*15.0*
HM resolution1	*15.0*
Ion energy 1	*0.0 - 1.0 eV*
Collision entrance	*±2 V*
Collision Energy	*set in scan function editor*
Collision exit	*0-3V*
LM resolution2	*15.0*
HM resolution2	*15.0*
Multiplier	650V

Data Acquisition

Overview

NeoLynx operates in an automated MassLynx environment, and data acquisition is usually achieved *via* the **Sample List** using some form of automated sample introduction. It is possible to acquire data manually for post-processing in a batch fashion. Multiple Sample Lists can be generated and queued up on the mass spectrometer. This manual has been written with a view to all the data acquisition and processing being performed from the Sample List.

Projects

MassLynx utilizes a project structure that has been designed to assist laboratories in GLP compliance. All acquisition settings files, raw data files, Sample Lists etc. are stored within a project. The Project structure is particularly useful for the NeoLynx application, as it is conceivable that a large number of samples will be analyzed on a daily basis.

It is strongly recommended that a **Master** project is created for each laboratory containing the basic information for the control of the instrumentation and data acquisition. The master project can then be used as a template for the creation of individual projects. It may be appropriate to create a project on a daily basis, as this will make data archiving and retrieval simpler.

MassLynx comes with some predefined projects NeoLynx_Research.pro and Default.pro are the two relevant to metabolite investigation. NeoLynx.pro is an example metabolite investigation project, only available when NeoLynx is installed. Default.pro is the default project where all data are stored until a new project has been selected or created.

For more information on projects see the **Getting Started** section of the MassLynx NT User Guide.

WHEN CREATING A NEW PROJECT, THE PROJECT NAME MUST NOT CONTAIN SPACES**.**

Calibration Note

If the instrument is recalibrated, the new calibration experiment should be performed in the <MASTER.PRO> project, used as the project template. In this way the calibration is automatically included in any new project created from the template.

ROOT DIRECTORY

The project root directory (<PROJECT_NAME>.PRO) contains seven sub-directories of which **Acqudb**, **Data** and **Sampledb** are the most important. The root directory also contains the NeoLynx Results files generated after NeoLynx processing of a Sample List. These files are discussed in more detail later.

DATA

This directory contains all the raw data directories associated with a particular project. Each raw directory contains the raw mass spectral information and any saved mass spectral processes. The folders are given the name of the acquisition with the format: <datafilename>.RAW.

SAMPLEDB

This directory contains the sample list files (*.SPL) that contain the run summary information. It is recommended that an individual SPL file is created for each batch (approximately one plate or 100 samples) as the SPL filename is used as a prefix for the NeoLynx Results files generated by the NeoLynx processing.

ACQUDB

This directory contains all the instrument control files used in the data acquisition. For some files, different extensions will be used depending on the type of interface card selected when the software was installed (TDA or Ethernet)

mass spectrometer tuning	*.dbf (TDAT) or *.ipr (Ethernet)
mass spectrometer acquisition	*.mdb (TDAT) or *.exp (Ethernet)
HPLC pump control	*.h11 for HP1100
	*.gil for Gilson
	*.w60 for Waters 600
	*.w27 for Waters 2700
	*.w29 for Waters 2790
Calibration files	*.cal

Sample Lists

The **Sample List** is part of the top level MassLynx screen. NeoLynx uses it to prepare a list of samples for analysis. The list of samples is set up using a spreadsheet style editor, which can be tailored to suit different requirements.

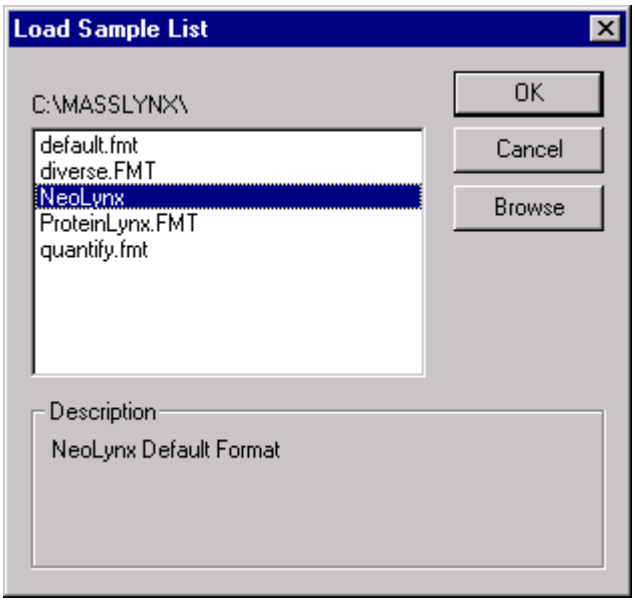


Figure 2.4 The Load Sample List Format dialog.

A NeoLynx Sample List format is supplied with the software. A Sample List format is a definition of the columns to be used in a Sample List. To use the NeoLynx format select **Samples, Format** from the **Samples List** menu, select **NeoLynx** from the dialog displayed (Figure 2.4) and press the **OK** button. See the MassLynx NT User Guide for details.

Propagation of the sample list is available to input large numbers of samples, with incremental descriptors, into the Sample List. A copy of a typical, propagated sample list for this application is shown in Figure 2.5

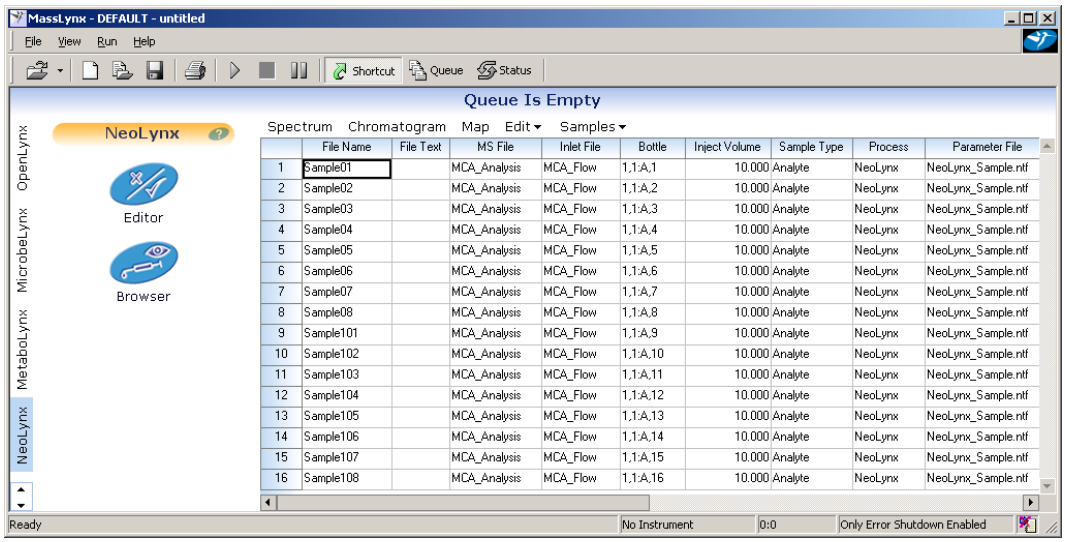


Figure 2.5 Sample List Editor

Full details on editing the sample list can be found in the **Sample List** section of the MassLynx NT User Guide. The parameters used in the Sample List in this example are:

File Name The raw data filename under which the data will be stored.

File Text The text that will be appended to each file. This may be used to contain patient details.

MS File The **Scan Function Editor** file that will be used to control the mass spectrometer during the data acquisition.

Inlet File The **Inlet Control File** that will be used to control the LC pump and autosampler.

Bottle The location of the sample in the autosampler tray.

Inject Volume The volume of sample to be injected in microlitres. It is recommended that the injection loop is over-filled for the best reproducibility. E.g. for a 20µL loop, inject 25-30µL.

Sample Type The type of sample. This is used to differentiate the different sample types when processing data. Available Sample Types are **Standard**, **Blank**, **QC** and **Analyte**. If no sample type is entered or Blank is selected then the sample will be processed as an analyte. The Sample Type definition is assigned when data is processed not acquired.

Parameter File The name of the NeoLynx Test File (*.ntf) to use.

Process Parameters In NeoLynx 3.2 and 3.3 the parameters '4 4' had to be entered in this column. In NeoLynx 4.0, this column must be empty.

Process The type of processing that will be performed on the data. In this case it should be NeoLynx.

For the **MS File**, **Inlet File**, **Sample Type**, **Process** and **Parameter File** fields files created and saved in the appropriate directories of the current project can be included in the sample list by double clicking on a cell and selecting the file from the drop down list displayed. See the MassLynx NT User Guide for further details.

If the file required is in another project then click on a cell and enter the full path name.

If any of the required columns do not appear in the sample list select **Samples, Format, Customize,** or right click on the sample list and from the menu select **Customize Display**; check the boxes for the required fields.

The columns labeled Spare1 to Spare5 in the Sample List can be used to enter extra information. This information can be used for variables in rule calculations or for printing on reports.

Once the Sample List has been prepared, it must be saved (**File**, **Save** or **Save As**) before continuing.

The name of the Browser Report file generated is based on the Sample List Name. E.g. Sample List Neo1.SPL will create a Browser Report file called Neo1_001.nrf. It is therefore essential that Sample Lists are saved with different names to ensure that Browser Report files are not overwritten. **The Sample List name must not contain spaces.**

To Start a Sample List Run

Select **Run, Start** from the main MassLynx menu or press the  toolbar button. This will display the dialog box shown in Figure 2.6.

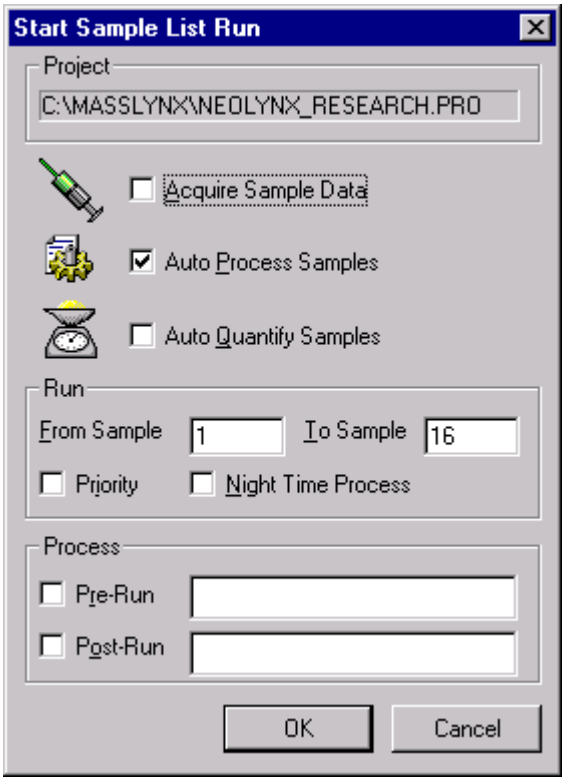


Figure 2.6 The Start Sample List Run dialog for processing only (Acquire Data box not checked)

Project This box shows where the acquired data will be stored, or from which directory the previously acquired data will be retrieved for processing. If this is incorrect press the **Cancel** button and select the correct project before starting the Sample List run again (see the **Getting Started** section of the MassLynx NT User Guide).

Acquire Sample Data	Specifies whether data is to be acquired. Do not check this box if only post-processing of previously acquired data is to be performed.
Auto Process Samples	Specifies whether data is to be processed. Checking both this box and the **Acquire Sample Data** box will acquire and process the data automatically. Do not check this box if only data acquisition is to be performed.
Auto Quantify Samples	The box is used to automatically Quantify the results and is not applicable to the NeoLynx process
Run from Sample *n* To Sample *m*	These boxes automatically default to the first and last samples in the current Sample List. It is possible to alter these values, to select a smaller range, by typing new values into the relevant boxes, or to highlight a range of samples in the Sample List with the mouse before displaying the dialog.
Priority	Checking this box will flag the Sample List as a priority process and put it above all non-priority lists in the queue.
Night Time Process	Check this box if the sample list is to be run at night.
Process	Allows routines to be executed before, during and after the Sample List has been run. For the NeoLynx application, there are no pre- or post-run processes.
OK	Pressing will add the Sample List to the Sample List queue on the mass spectrometer. The queue appears at the bottom of the MassLynx screen and allows the user to delete, prioritize and pause Sample Lists. See the **Getting Started** section of the MassLynx NT User Guide for more details.

Scan Function Editor

MassLynx allows for up to eight separate scan functions to be acquired. It is recommended that data acquired from the mass spectrometer is separated into discrete functions, that relate to a specific class of metabolites and their related internal standards. It is possible to include full-scan MCA functions with MRM functions. For a full explanation of the **Function List Editor**, see the MassLynx NT Guide to Data Acquisition.

Scan Functions for Butyl Ester Derivatives

The following experiments have been described in the literature, and are included as a guide only. It is recommended that tuning and collision conditions be optimized for each compound on each instrument.

Compound Class	Function	Mass range	Cone Voltage	Collision Energy	Polarity	Scan Time
Acylcarnitines	Parents of *m/z* 85.0	*m/z* 200 - 500	35	25	+ve	3.0s
Neutral and acidic amino acids	Neutral Loss of 102.1 Da	*m/z* 140 - 270	25	12	+ve	1.5s
Basic amino acids	neutral Loss of 119.1 Da	*m/z* 160 - 270	28	25	+ve	1.0s
Glycine and Alanine	Neutral Loss of 56.0 Da	*m/z* 120 - 160	22	8	+ve	0.5s
Arginine	Neutral Loss of 161.1 Da	*m/z* 220 - 240	40	35	+ve	0.2s
Argininosuccinic acid	Daughters of *m/z* 459.4	*m/z* 60 - 150	35	25	+ve	1.0s

Full-Scan Acquisitions

A typical acquisition sequence from the MassLynx scan editor is shown in Figure 2.7.

There are two functions to be acquired in full scan MCA mode.

1. Scans for the Parent ion of *m/z* 85.0 (acylcarnitines) for 30 seconds, followed by:

2. A scan for a constant Neutral Loss of 102.1 Da (amino acids) for 30 seconds.

In this analysis, acylcarnitines and amino acids are detected sequentially. The exact timing sequence will be determined by

(i) the size of the injection.

(ii) the flow rate of the mobile phase.

(iii) the length of the transfer tubing from the injector to the ion source.

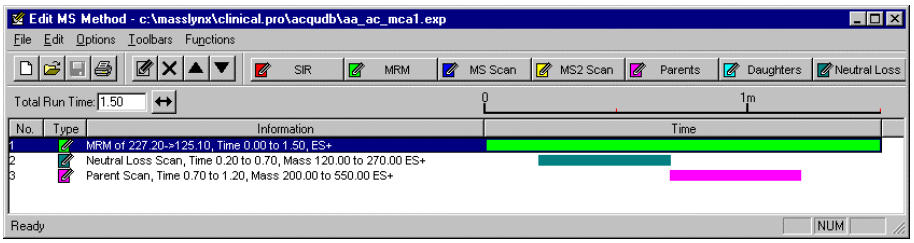


Figure 2.7 The scan function editor for sequential full-scan MCA acquisition

The acylcarnitines and amino acids can also be detected simultaneously in a looped experiment, as in Figure 2.8

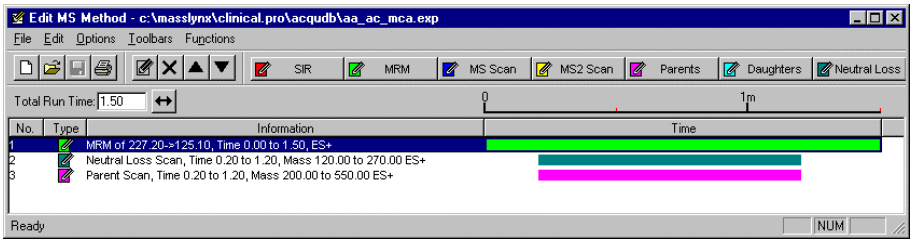


Figure 2.8 The scan function editor for simultaneous full-scan MCA acquisition

Parameters currently used for these scan functions are shown in Figure 2.9, Figure 2.10 and Figure 2.11, along with Collision gas pressure (Ar) = 2.0×10^{-3} mbar.

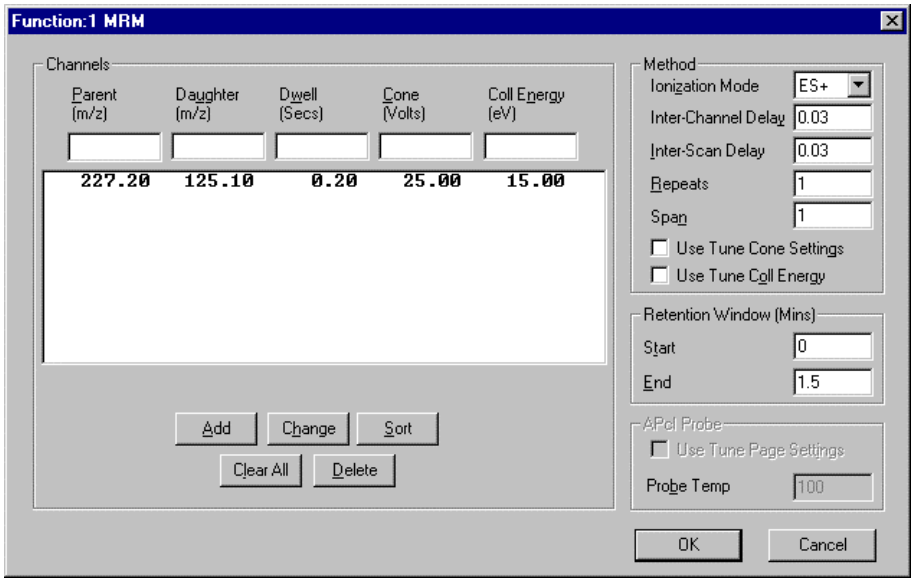


Figure 2.9 Scan function details for MRM scan (Function 1)

This will allow for the injection profile to be monitored for each sample.

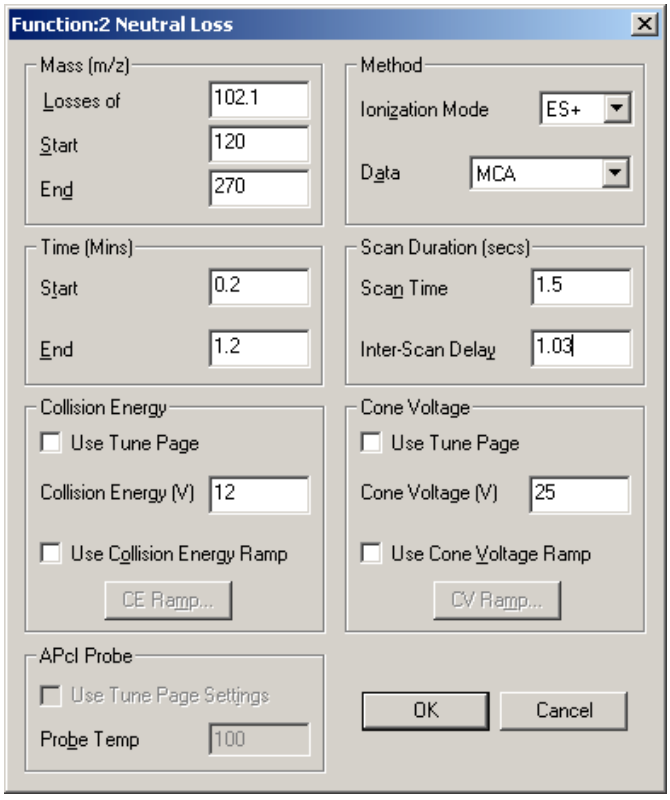


Figure 2.10 Scan function details for constant neutral loss scan (Function 2)

This will give a mass spectrum of all the butylated amino acids present in the sample.

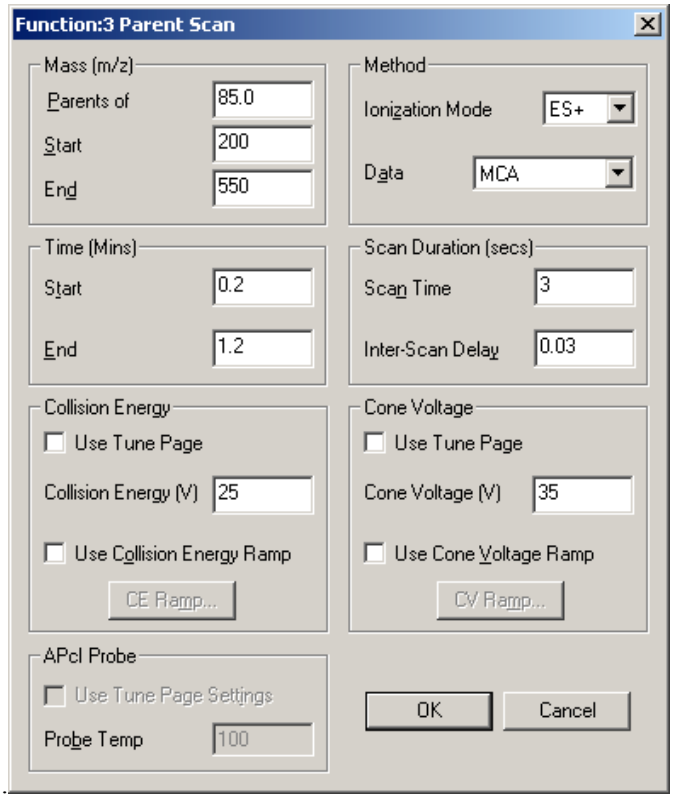


Figure 2.11 Scan function details for precursor ion scan (Function 3).

This will give a mass spectrum of the butylated acylcarnitines present in the sample.

In order to maintain mass spectral resolution when scanning in Parent or Constant Neutral Loss modes, a scan speed of between 100 and 150 *m/z* per sec is recommended.

MRM Acquisitions

If the analytical method only requires specific analytes to be monitored then a multiple reaction monitoring sequence should be used. Each function within the MassLynx scan editor is capable of monitoring up to 32 transitions. Again, it is a requirement of NeoLynx that each function is dedicated to one class of analytes and the associated internal standards.

Two functions used for the specific detection of acylcarnitines and amino acids are shown below. The number of channels actually used in a particular analysis will depend on the analytes of interest and the availability of internal standards.

Function 1 - For butylated acylcarnitines

Function type: MRM of 28 channels

Chain Reaction	Dwell	Cone Volt	Col.Energy	Analyte
1 : 218.20 > 85.00	0.05	35	25	Free carnitine
2 : 221.20 > 85.00	0.05	35	25	d_3-carnitine (i.s.)
3 : 260.20 > 85.00	0.05	35	25	C_2-carnitine
4 : 263.20 > 85.00	0.05	35	25	d_3-C_2-carnitine (i.s.)
5 : 274.20 > 85.00	0.05	35	25	C_3-carnitine
6 : 288.20 > 85.00	0.05	35	25	C_4-carnitine
7 : 300.20 > 85.00	0.05	35	25	$C_{5:1}$-carnitine

8 : 302.20 > 85.00	0.05	35	25	C_5-carnitine
9 : 316.20 > 85.00	0.05	35	25	C_6-carnitine
10 : 318.20 > 85.00	0.05	35	25	C_5-OH-carnitine
11 : 342.30 > 85.00	0.05	35	25	$C_{8:1}$-carnitine
12 : 344.30 > 85.00	0.05	35	25	C_8-carnitine
13 : 347.30 > 85.00	0.05	35	25	d_3-C_8-carnitine (i.s.)
14 : 370.30 > 85.00	0.05	35	25	$C_{10:1}$-carnitine
15 : 372.30 > 85.00	0.05	35	25	C_{10}-carnitine
16 : 400.30 > 85.00	0.05	35	25	C_{12}-carnitine
17 : 426.40 > 85.00	0.05	35	25	$C_{14:1}$-carnitine
18 : 428.40 > 85.00	0.05	35	25	C_{14}-carnitine
19 : 444.40 > 85.00	0.05	35	25	C_{14}-OH carnitine
20 : 454.40 > 85.00	0.05	35	25	$C_{16:1}$-carnitine
21 : 456.40 > 85.00	0.05	35	25	C_{16}-carnitine
22 : 459.40 > 85.00	0.05	35	25	d_3-C_{16}-carnitine (i.s.)
23 : 470.40 > 85.00	0.05	35	25	$C_{16:1}$-OH-carnitine
24 : 472.40 > 85.00	0.05	35	25	C_{16}-OH-carnitine
25 : 480.40 > 85.00	0.05	35	25	$C_{18:2}$-carnitine
26 : 482.40 > 85.00	0.05	35	25	$C_{18:1}$-carnitine
27 : 496.40 > 85.00	0.05	35	25	C_{18}-carnitine
28 : 498.40 > 85.00	0.05	35	25	$C_{18:1}$-OH-carnitine

Function 2 - For butylated amino acids

Function type: MRM of 23 channels

Chain Reaction	**Dwell (secs)**	**Cone Volt**	**Col.Energy**	**Analyte**
1 : 132.10 > 30.00	0.05	25	12	Gly
2 : 134.10 > 32.00	0.05	25	12	$^{13}C^{15}N$-Gly (i.s.)
3 : 146.10 > 44.00	0.05	25	12	Ala
4 : 150.10 > 48.00	0.05	25	12	d_4-Ala (i.s.)
5 : 162.10 > 60.00	0.05	25	12	Ser
6 : 172.10 > 70.00	0.05	25	12	Pro
7 : 174.10 > 72.00	0.05	25	12	Val
8 : 176.10 > 74.00	0.05	25	12	Thr
9 : 182.10 > 80.00	0.05	25	12	d_8-Val (i.s.)
10 : 188.10 > 86.00	0.05	25	12	Leu/Ile
11 : 191.10 > 89.00	0.05	25	12	d_3-Leu (i.s.)
12 : 206.20 > 104.10	0.05	25	12	Met
13 : 209.20 > 107.10	0.05	25	12	d_3-Met (i.s.)
14 : 212.20 > 110.10	0.05	25	12	His
15 : 215.20 > 113.10	0.05	25	12	Cit
16 : 222.20 > 120.10	0.05	25	12	Phe
17 : 227.20 > 125.10	0.05	25	12	d_5-Phe (i.s.)
18 : 238.20 > 136.10	0.05	25	12	Tyr
19 : 242.20 > 140.10	0.05	25	12	d_4-Tyr (i.s.)
20 : 246.20 > 144.10	0.05	25	12	Asp
21 : 260.20 > 158.10	0.05	25	12	Glu
22 : 261.20 > 159.10	0.05	25	12	Trp
23 : 263.20 > 161.10	0.05	25	12	d_3-Glu (i.s.)

This acquisition would again be prepared in the Scan Function Editor and would be similar to that seen in Figure 2.12

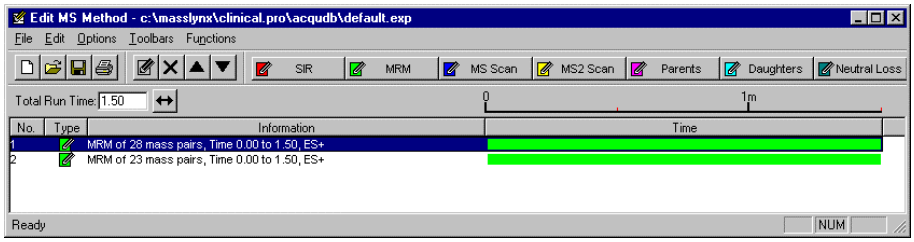


Figure 2.12 Scan function editor for MRM acquisition

In the MRM analysis shown, the two functions are acquired in parallel, this means that all the channels for the first function are acquired, immediately followed by the channels for the second function, and so on. On completion of the acquisition of all the functions, the acquisition returns to the start of the first function. The details of the first function are shown in Figure 2.13.

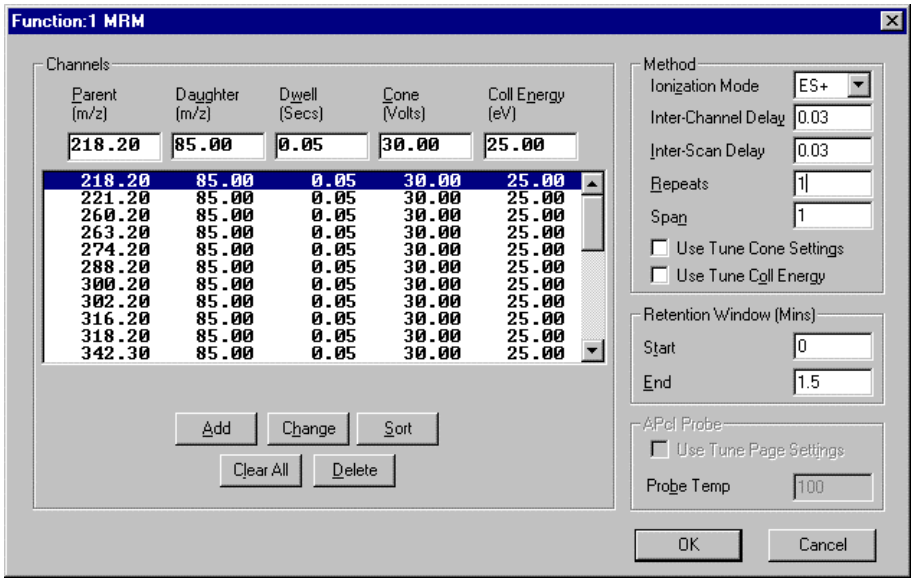


Figure 2.13 MRM function details

When using an MRM acquisition, the data acquisition is initiated as soon as the sample has been injected into the mobile phase passing into the electrospray source of the mass spectrometer. The reaction chromatograms for each function are stored in a single data directory, each in a separate data file. The total ion chromatogram for the first function (acylcarnitines in this instance) is shown in Figure 2.14.

As can be seen from Figure 2.14, the analyte passes from the injector to the ion source in less than 20 seconds, lasting for approximately 30 seconds. In this time, each reaction channel is tested approximately 10 times, and is sufficient to provide good chromatographic definition. (Note a chromatogram is acquired even though no separation takes place). The benefit of this type of acquisition is that the mobile phase in the mass spectrometer may flow somewhat quicker (up to 40 µL/min) than in the full-scan MCA acquisition (up to 10 µL/min), and the inter-sample time may be reduced.

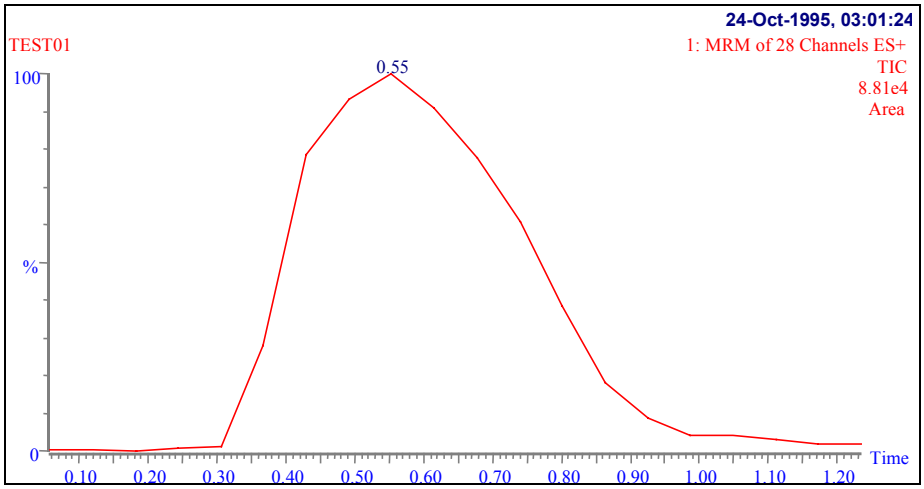


Figure 2.14 Total ion chromatogram for acylcarnitine MRM function (time abscissa)

In both full scan and MRM acquisitions, it is recommended that the sample bolus is sampled at least 7 times as it passes into the ion source. This is to average out any dispersion (and 'chromatography') that may have occurred in the tubing between the injection loop and the mass spectrometer.

Chapter 3 Data Processing with NeoLynx

Table of Contents

Figures

Overview

The intensity of a mass spectral peak in electrospray ionization is proportional to the concentration of the compound in the analysis. NeoLynx operates by calculating the ratio of the intensity of the mass spectral peak for the analyte to the intensity of the mass spectral peak for a pre-defined internal standard (e.g. d_5-phenylalanine). If the concentration of the internal standard is known, then the concentration of the analyte may be calculated by simply multiplying the peak intensity ratio by the concentration of the internal standard.

In addition to the calculation of simple peak intensity ratios, and thus analyte concentrations, it is also possible to monitor individual peak intensities so as to monitor the performance of the complete assay (extraction, derivatization and mass spectral analysis).

In recent publications, there has been considerable discussion relating to the utility of complex ratios of analytes e.g. [Phe]/[Tyr]. It is possible to use NeoLynx to perform such calculations.

All of the values reported by NeoLynx are compared to a set of maxima and minima that are specific for each test performed. If the calculated peak intensity or peak intensity ratio falls outside the acceptable limits, a user-defined message is returned.

NeoLynx uses a number of specific files for processing and viewing results.

NeoLynx Test Files (*.ntf) These files contain the ratio test information, spectral (and chromatogram) processing information and test file modification history for audit purposes.

NeoLynx Result Files (*.nrf) These files contain the results for the specified Sample List, including the mass spectral and chromatographic data, the test results and information about the NeoLynx Test File used to generate it.

NeoLynx Report Scheme (*.nrs) These files define the reporting options used by the NeoLynx Browser for generating printed and electronic summary reports.

The NeoLynx Test Files and the NeoLynx Report Schemes are controlled by the MassLynx Security system and may not be edited or saved without appropriate permissions. The NeoLynx Test Files and NeoLynx Results Files are also encrypted to prevent access by unauthorized personnel from a non-MassLynx application.

Introduction

NeoLynx is an integral part of MassLynx that has been designed for the post-processing of data acquired in a neonatal screening-type experiment.

NeoLynx requires that the analytes and their associated internal standards have both been acquired in the same mass spectrometric function. Regardless of the type of data (full-scan MCA, continuum or MRM), NeoLynx processes the data to give a mass measured or combined centroided spectrum. NeoLynx automatically recognizes the type of data to be processed.

Processing information is stored with each sample in the NeoLynx Results file.

Once the centroided data has been prepared, NeoLynx calculates ratios of certain peaks within the processed spectrum and tests the derived ratios against limits defined in the NeoLynx Test file. A report is generated that contains a summary of all the spectral information, processing data, tests performed and the results of the ratio comparisons.

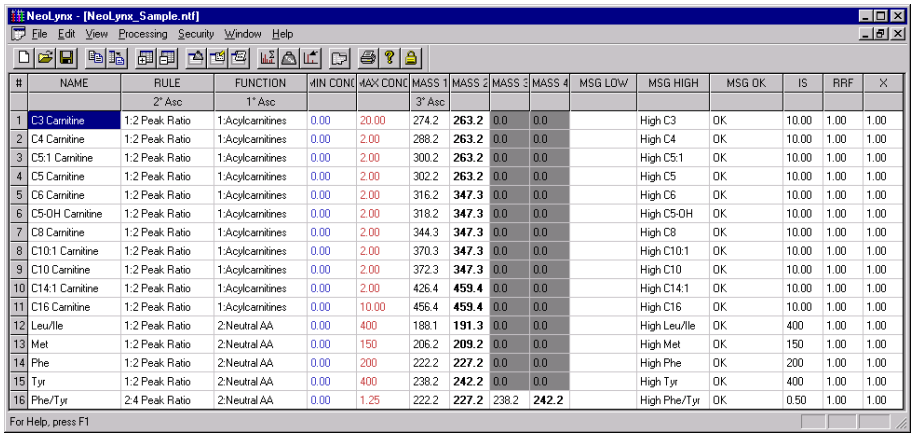
The NeoLynx Results file is saved to the root directory of the current project (*.PRO) and can be viewed using the NeoLynx Browser (see the NeoLynx Browser chapter).

The name of the NeoLynx Results file generated is based on the Sample List Name. E.g. Sample List Neo1.SPL will create a Browser Report file called Neo1.nrf. It is therefore essential that Sample Lists be saved with different names to ensure that Browser Report files are not overwritten. **The Sample List name must not contain spaces**.

All the information required to calculate the peak intensity ratios and to generate the report is defined in the **NeoLynx Test File**.

NeoLynx Test File Editor

The **NeoLynx Test File Editor** can be accessed from the top level MassLynx screen by selecting **NeoLynx, Editor** from the MassLynx shortcut Bar.



#	NAME	RULE	FUNCTION	MIN CONC	MAX CONC	MASS 1	MASS 2	MASS 3	MASS 4	MSG LOW	MSG HIGH	MSG OK	IS	RRF	X
		2° Asc	1° Asc			3° Asc									
1	C3 Carnitine	1:2 Peak Ratio	1:Acylcarnitines	0.00	20.00	274.2	**263.2**	0.0	0.0		High C3	OK	10.00	1.00	1.00
2	C4 Carnitine	1:2 Peak Ratio	1:Acylcarnitines	0.00	2.00	288.2	**263.2**	0.0	0.0		High C4	OK	10.00	1.00	1.00
3	C5:1 Carnitine	1:2 Peak Ratio	1:Acylcarnitines	0.00	2.00	300.2	**263.2**	0.0	0.0		High C5:1	OK	10.00	1.00	1.00
4	C5 Carnitine	1:2 Peak Ratio	1:Acylcarnitines	0.00	2.00	302.2	**263.2**	0.0	0.0		High C5	OK	10.00	1.00	1.00
5	C6 Carnitine	1:2 Peak Ratio	1:Acylcarnitines	0.00	2.00	316.2	**347.3**	0.0	0.0		High C6	OK	10.00	1.00	1.00
6	C5-OH Carnitine	1:2 Peak Ratio	1:Acylcarnitines	0.00	2.00	318.2	**347.3**	0.0	0.0		High C5-OH	OK	10.00	1.00	1.00
7	C8 Carnitine	1:2 Peak Ratio	1:Acylcarnitines	0.00	2.00	344.3	**347.3**	0.0	0.0		High C8	OK	10.00	1.00	1.00
8	C10:1 Carnitine	1:2 Peak Ratio	1:Acylcarnitines	0.00	2.00	370.3	**347.3**	0.0	0.0		High C10:1	OK	10.00	1.00	1.00
9	C10 Carnitine	1:2 Peak Ratio	1:Acylcarnitines	0.00	2.00	372.3	**347.3**	0.0	0.0		High C10	OK	10.00	1.00	1.00
10	C14:1 Carnitine	1:2 Peak Ratio	1:Acylcarnitines	0.00	2.00	426.4	**459.4**	0.0	0.0		High C14:1	OK	10.00	1.00	1.00
11	C16 Carnitine	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	456.4	**459.4**	0.0	0.0		High C16	OK	10.00	1.00	1.00
12	Leu/Ile	1:2 Peak Ratio	2:Neutral AA	0.00	400	188.1	**191.3**	0.0	0.0		High Leu/Ile	OK	400	1.00	1.00
13	Met	1:2 Peak Ratio	2:Neutral AA	0.00	150	206.2	**209.2**	0.0	0.0		High Met	OK	150	1.00	1.00
14	Phe	1:2 Peak Ratio	2:Neutral AA	0.00	200	222.2	**227.2**	0.0	0.0		High Phe	OK	200	1.00	1.00
15	Tyr	1:2 Peak Ratio	2:Neutral AA	0.00	400	238.2	**242.2**	0.0	0.0		High Tyr	OK	400	1.00	1.00
16	Phe/Tyr	2:4 Peak Ratio	2:Neutral AA	0.00	1.25	222.2	**227.2**	238.2	**242.2**		High Phe/Tyr	OK	0.50	1.00	1.00

Figure 3.1 NeoLynx Test File Editor

Warning

NeoLynx uses rule editor files in which the fields are separated by commas. In certain countries it is common to use a comma instead of a decimal point, and a period as a thousand separator (e.g. 1.0 → 1,0 and 1,000.23 → 1.000,23). MassLynx is configured with the computers regional settings appropriately set in the Control Panel. This must not be changed under any circumstances.

Security

If the User is not authorized to modify the NeoLynx Test Files, or if the NeoLynx Test File is opened without MassLynx Security running, it will not be possible to modify and save the file. In addition all relevant buttons will be grayed out.

The Test Editor Toolbar

The Toolbar is displayed at the top of the test editor window and allows some common operations to be performed with a single click of the appropriate Toolbar button.

 Press to create a new NeoLynx Test file.

 Press to open an existing NeoLynx Test file.

 Press to save the current NeoLynx Test file.

 Press to copy the selected part of the table.

 Press to paste the previously copied data onto the table. **Note**: The Paste area must be the same size as the copied area.

 Press to add a test.

 Press to delete a test.

 Press to view the Mandatory Mass table.

 Press to view the Function Name table.

 Press to view the Rule Definition table.

 Press to view the NeoLynx Combine parameters.

 Press to view the NeoLynx Mass Measure parameters.

 Press to view the NeoLynx Peak Detect parameters.

 Press to view the Processing Options.

 Press to print the current NeoLynx Test file.

 Press to display the About box.

Getting Started

To Create a NeoLynx Test Editor File

Press the  Toolbar button, or select **New** from the Test Editor **File** menu. A new blank table is created.

Enter the required test data as described later in this chapter.

Press the  toolbar button, or select **Save** or **Save As** from the Test Editor **File** menu. Enter a name for the new Test Editor file and press **OK**.

The Test Modification History dialog will be displayed.

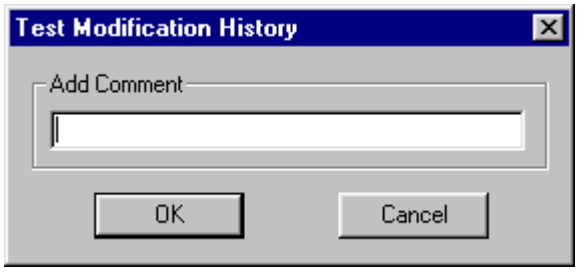


Figure 3.2 Test Modification History dialog

This dialog is displayed when a file is saved and is used to keep a record of changes made to a test file. Comments added here can be viewed by selecting **Modification History** from the NeoLynx Test Editor **View** menu.

Enter comments as required (up to 50 characters) and press **OK**.

To Open an Existing Test Editor File

Press the  Toolbar button, or select **Open** from the Test Editor **File** menu.

Select the required NeoLynx Test Editor file (*.ntf) and press **Open**.

To Convert a Rule Editor File

NeoLynx V3.2 and V3.3 Rule files (*.rle) created in previous version of NeoLynx can be converted into NeoLynx Test Files (*.ntf).

Press the  Toolbar button, or select **Open** from the Test Editor **File** menu. NeoLynx Test Files(*.ntf) are the default file types. Click on the  arrow, at the end of the **Files of type** box and select NeoLynx Rules Files (*.rle).

Select the required file and press **Open** to display the **Convert RLE File** dialog.

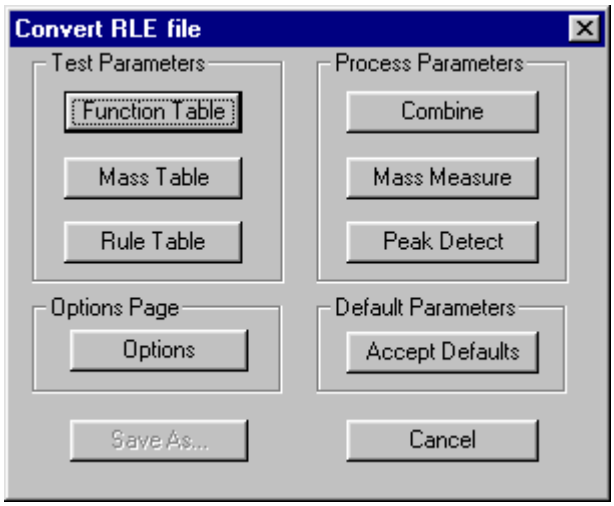


Figure 3.3 Convert RLE file dialog

Before the table can be saved the **Combine**, **Mass Measure** and **Peak Detect** parameters must be defined. Press the relevant buttons and enter values in the dialogs displayed or press the **Accept Defaults** button to use the default values listed below. Files cannot be saved until all the required fields have been entered.

Combine

Average = 5:10
Peak separation = 1.000
Subtract = 2:3
X = 1.000

Peak Detection

Peak Selection =
Largest Peak in Window

Mass Window (a.m.u.) = 1.00

Mass Measure

Background subtract selected
Polynomial order = 30
Below curve (%) = 10.00
Smooth selected
Peak width (Da) = 1.00
Number of smooths = 2
Savitzky Golay selected
Min peak width at half height (channels) = 2
Top selected

The following parameters on the Options dialog are also defined.

Process Options tab

Pass Message = All Tests Within Limits
Output File = Up Issue
Create MassLynx Process selected

Variables tab

Mass 1 = 111.1	*X=3*
Mass 2 = 222.2	*s1 = 10*
Mass 3 = 333.3	*s2 = 20*
Mass 4 = 444.4	*s3 = 30*
IS Conc = 1	*s4 = 40*
RRF = 2	*s5 = 50*

The **Function Table**, **Mass Table**, **Rule Table** and **Options** parameters can also be defined by pressing the relevant button and entering values in the dialogs displayed.

All dialogs are described in detail later in this chapter. Values can be modified after the table has been saved.

When the required values have been defined press the **Save As** button, enter the name of the files and press **Save**.

The Test Modification History dialog is displayed, enter comments as required (up to 50 characters) and press **OK**.

Test Modification History

The **Test History** (Figure 3.4) provides an audit trail for NeoLynx Test File modifications and is displayed by selecting **Modification History** from the Test Editor **View** menu.

The Modification History contains:

- the date and time of modification
- the action performed
- the name of the operator logged on to MassLynx
- a comment entered in the Test Modification History dialog.

The modification history is stored as part of the NeoLynx Test file.

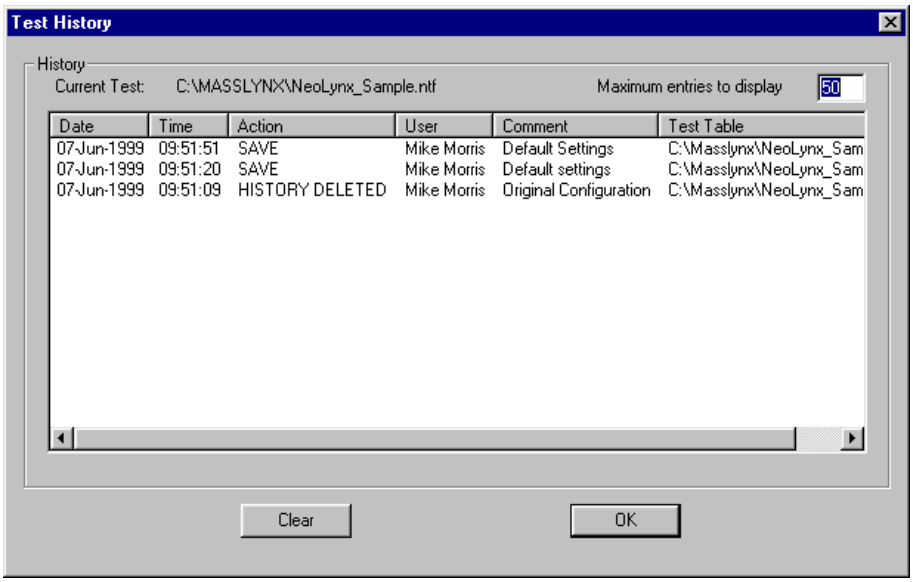


Figure 3.4 NeoLynx Test History dialog

Processing Options

The Process Options dialog may be accessed by pressing the  toolbar button or selecting **Options** from the Test Editor **Processing** menu. This dialog contains information relating to the automatic generation of printed and electronic reports.

Print Options Page

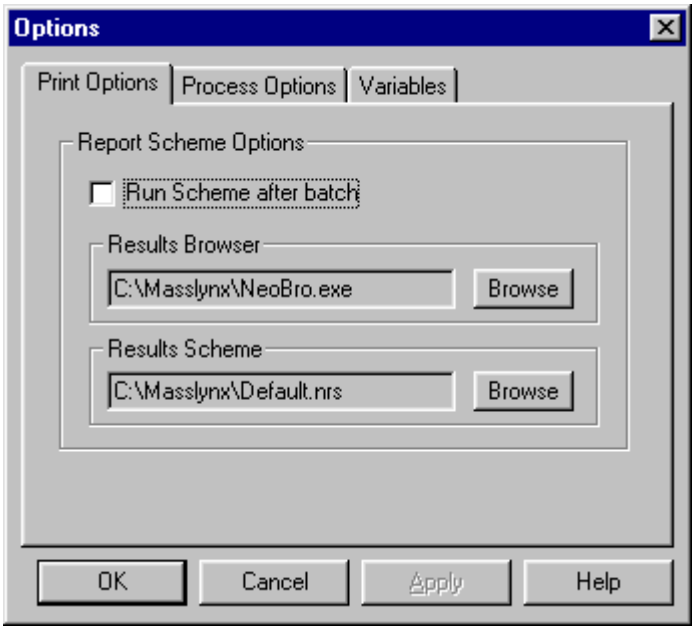


Figure 3.5 The Processing Options: Print Options page

Run Scheme after batch Check this box to generate printed reports and electronic reports on completion of each Sample List (if NeoLynx processing is specified).

Results Browser This defines the location of the NeoLynx Browser program. Press the **Browse** button, locate the NeoBro.exe program, highlight it and press **Open** to define the path. By default this is the C:\MassLynx directory.

Report Scheme This defines the location of the NeoLynx Report Scheme used to generate the printed and electronic reports. Press the **Browse** button, locate the required *.nrs file, highlight it and press **Open** to define the path. By default this is the C:\MassLynx directory. See the NeoLynx Browser chapter for details on Report Schemes

Process Options Page

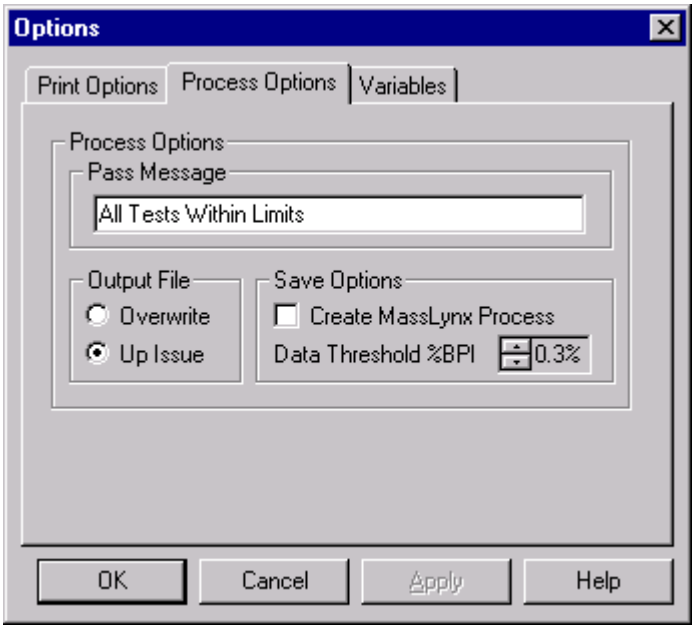


Figure 3.6 The Processing Options: Process Options page

Pass Message Enter the text to be displayed in the Browser Report if the results for all tests lie within normal limits.

Output File Select **Overwrite** or **Up Issue**. NeoLynx processes raw data files and creates a NeoLynx Results file whose name is based on the Sample List name.

If Overwrite is selected then any existing Results File with the same name will be overwritten.

If Up Issue is selected then the file name will have an issue number added to it (e.g. filename_001.nrf. Each time a report with the same name is generated this number is increased by 1. The maximum number of issues is 999, after which a warning message is generated.

Save Options In NeoLynx V4.0 the processed data and the processing parameters are stored in the NeoLynx Results File. To save this information as a process in the data file check the **Create MassLynx Process** box. This option is provided to provide back-compatibility with previous versions of NeoLynx, and is not required for NeoLynx 4.0 unless specified in the laboratory protocol.

Data Threshold %BPI

Use the arrows to enter a data threshold value. Any peak that is less than this percent of the base peak intensity will not be stored in the NeoLynx Results file. Range 0.0 to 3.0%. Setting a threshold value of 1.0% (depending on the noise of the system) can reduce the size of the NeoLynx Results File significantly. The threshold value does not affect the calculated data in the NeoLynx Results file, only the spectral display data.

Variables Page

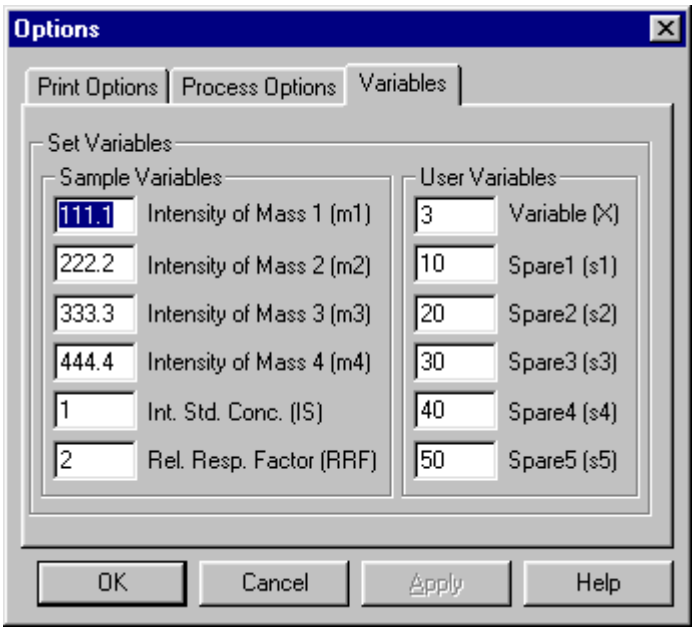


Figure 3.7 The Processing Options: Variables page

This dialog is used to define values for evaluating the results of a rule, and testing the mathematical validity of a rule expression. See Test on page 3-15 for more details.

Function Names Table

NeoLynx provides a feature where the names of the mass spectrometer's functions can be defined. This is for Test Editor display only purpose and does not relate interactively with the MS Method editor definitions. Once completed, the functions can be selected from a drop down list box for insertion into a NeoLynx Test File. To access the Function Names dialog press the toolbar button or select **Function Name Table** from the **View** menu.

It is possible to set the scaling for the spectra to be viewed in the NeoLynx Browser. For each function enter a mass and a nominated intensity (Percent). E.g. for consistency, it may be required to set the intensity of an internal standard peak to be 50% of the mass spectral scale. If these parameters are set to zero, default spectral scaling will be used.

The Function Names Table must be defined before the Mandatory Mass Table as Mandatory Masses cannot be defined without a Function.

Apart from the Function Name two other fields are available: -

Mass Defined as the "lock" mass in the spectrum.

Percent The percentage value at which the lock mass will be displayed.

To Add a Function Name

1. Enter a function **Number** and **Function** name.

2. Press the **Add** button, the details will be added to the table.

To Modify a Function Name

1. Position the cursor on the entry to be modified. This can be done by clicking on the field or by using the arrow keys.

2. The current details are displayed in the Number and Function fields. Change the function name as required.

3. Press the **Add** button and the entry will be updated in the table.

Note: If the function Number is changed then a new entry will be added to the table.

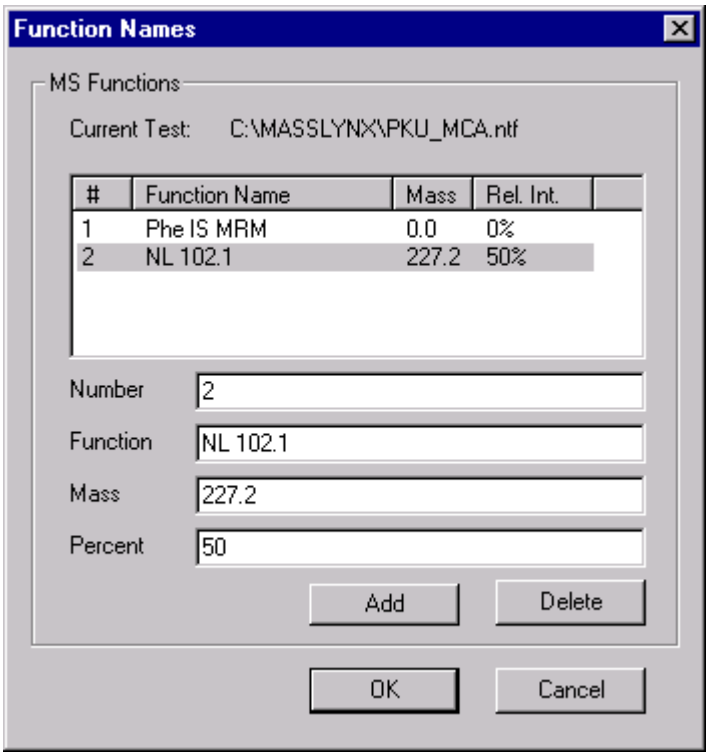


Figure 3.8 Function Names dialog

To Delete a Function Name

1. Position the cursor on the entry to be deleted. This can be done by clicking on any field in the row or by using the arrow keys.

2. Press the **Delete** button and the entry will be deleted from the table.

Mandatory Mass Table

To access the Mandatory Masses dialog (Figure 3.9) press the  toolbar button or select **View, Mandatory Mass Table**.

The Mandatory Mass Table is used to define peaks that **must** be found by the NeoLynx processing algorithms. For example, peaks corresponding to internal standard compounds should be present in each sample and labeled as mandatory.

Masses defined as mandatory are displayed in bold in the Test Editor table.

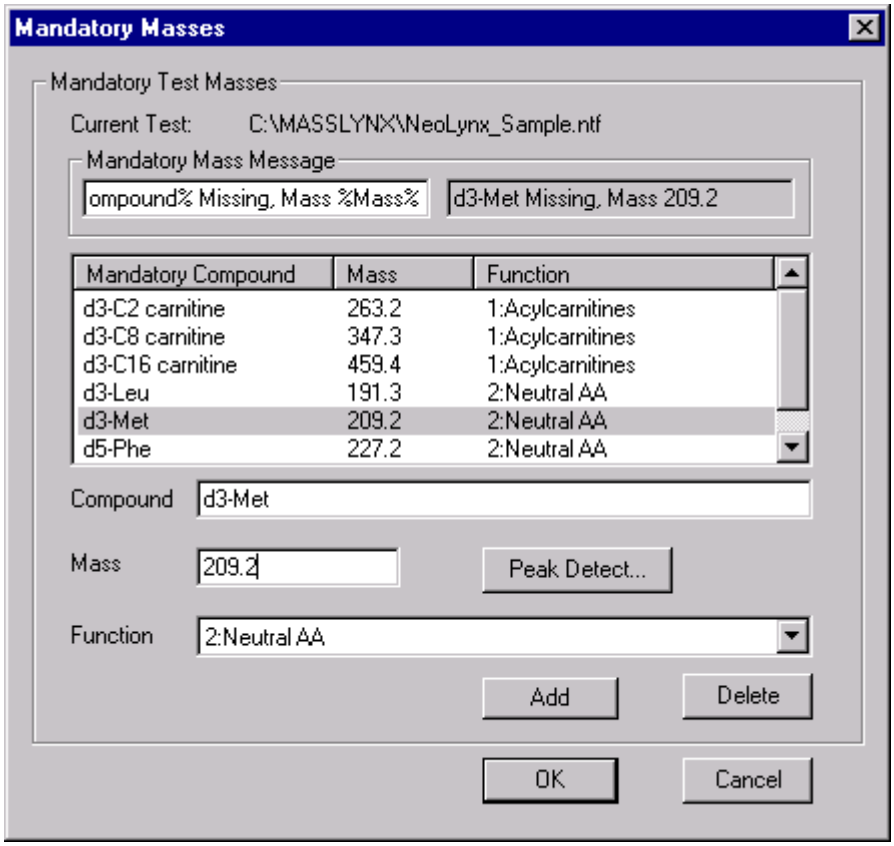


Figure 3.9 Mandatory Masses dialog

Peak Detect

A mandatory mass will only be defined as being missing if it falls below a threshold of the base peak intensity. This can be defined by selecting the **Peak Detect** button.

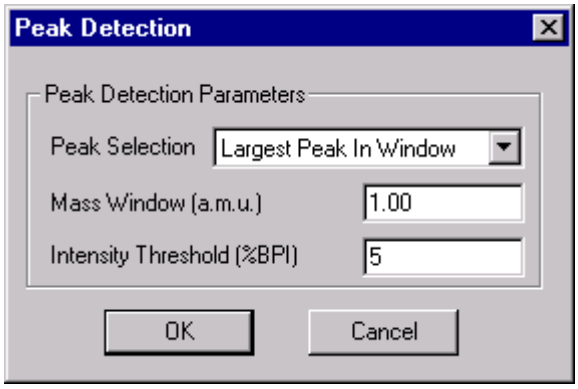


Figure 3.10 - Peak detection dialog for Mandatory Mass

Peak Selection Select **Largest Peak in Window** or **Nearest Peak to Centre** from the drop down list box.

Mass Window Enter the window, around the mandatory mass, to look for the peak in

Intensity Threshold Enter the percentage intensity of the base peak, below which the mandatory mass will be flagged as being absent.

If the processing parameters are correct there should only be one peak in the window of interest. The software will inspect the Mass Window looking for the largest peak or the peak nearest to the center. If the intensity of the peak is above the Intensity Threshold it will be marked as found, if not it will be marked as missing and the Mandatory Mass Message will be displayed.

Mandatory Mass Message

The Mandatory Mass Message is generated if a mandatory mass peak is not found. This message can be one of the following:

- A plain text message (e.g. Mandatory Mass Missing).

A message containing the name defined in the compound field. Enter %Compound% as in Figure 3.9 selecting **Mandatory Mass Table** from the pop up menu displayed. The Mandatory Mass Table dialog is displayed with the mass and function copied from the Test Editor table. Enter the compound name and press the **Add** button.

To Modify a Mandatory Compound Name

Position the cursor on the entry to be modified. This can be done by clicking on the field or by using the arrow keys.

The current details are displayed in the Compound, Mass and Function fields. Change the compound name as required.

Press the **Add** button and the entry will be updated in the table.

If the Mass or Function is changed then a new entry will be added to the table.

To Delete a Mandatory Mass

Position the cursor on the entry to be deleted. This can be done by clicking on any field in the row or by using the arrow keys.

Press the **Delete** button and the entry will be deleted from the table.

Rule Definition Table

To access the Rule Table dialog (Figure 3.11) press the  toolbar button or select **View, Rule Table** from the menu.

The Rule Definition Table (see Figure 3.11) is used to define the calculations performed for a rule.

The number of the Rule.

Note: Rules 0, 1 and 2 are pre-defined and correspond to the Rules defined in NeoLynx V3.3. For these three rules only the rule name, multiplier and units fields can be modified.

Rule A user-definable name that makes selection of the different rules in the NeoLynx Test Table possible from a drop-down menu with easy reference.

Concentration formula The equation used as the test on the mass spectral data. The result of this equation is tested against the **Min Conc** and **Max Conc** fields in the NeoLynx Test Editor table. Available variables: **m1**, **m2**, **m3** and **m4** for the masses of interest. Three variables (**IS**, **RRF**, **X**) are defined in the NeoLynx Test Editor table, and five variables (**s1**, **s2**, **s3**, **s4** and **s5**) are available from the Sample List fields Spare1 - Spare5. All mathematically valid equations may be used.

Units User-defined units used on reports.

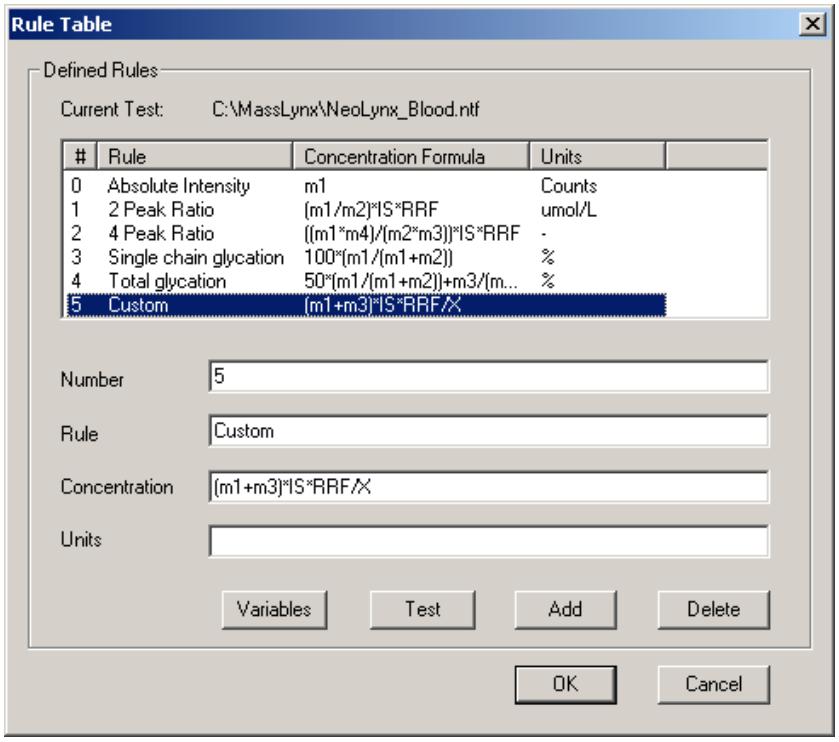


Figure 3.11 Rule Table dialog

To Add a Rule

1. Enter a rule **Number**, rule **Description**, **Concentration Formula** and **Units** value in the appropriate fields.

2. Press the **Add** button, the details will be added to the table.

To Modify a Rule

1. Position the cursor on the entry to be modified. This can be done by clicking on the field, , or by using the arrow keys.

2. The current details are displayed in the Number, Rule, Concentration and Units. Change the fields as required.

3. Press the **Add** button and the entry will be updated in the table.

Note: If the function Number is changed then a new entry will be added to the table.

To Delete a Rule

1. Position the cursor on the entry to be deleted. This can be done by clicking on any field in the row, or by using the arrow keys.

2. Press the **Delete** button and the entry will be deleted from the table.

Variables

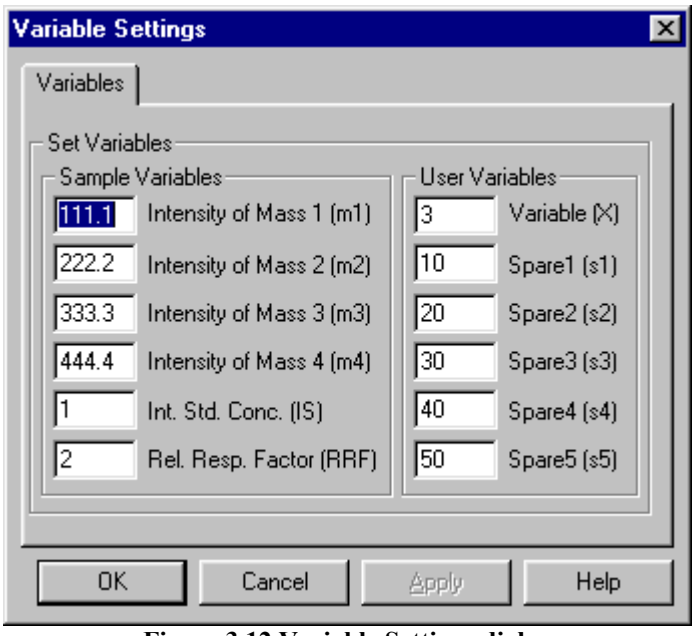


Figure 3.12 Variable Settings dialog

This dialog is used to define values for evaluating the results of a rule, and testing the mathematical validity of a rule expression. Enter values similar to the ones expected and press **OK**. Press the **Test** button on the Rule Table dialog to see the results of the equation.

The values used to derive the results saved in the NeoLynx Report file are those defined in the NeoLynx Test File table (IS, RRF and X columns) and/or the Sample List (Spare1-5).

Test

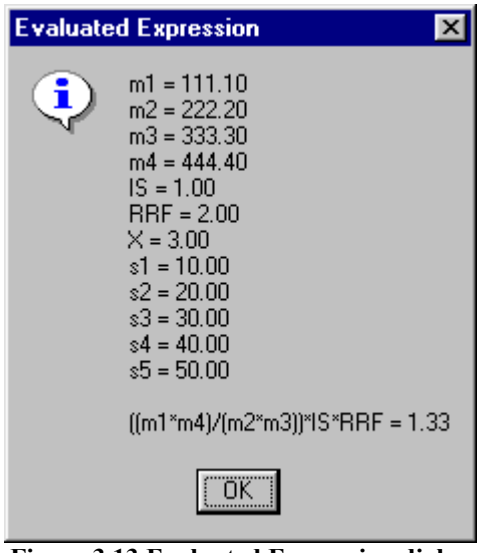


Figure 3.13 Evaluated Expression dialog

Press the **Test** button to check the results of a Rule. This operation tests the mathematical validity of the Ion Ratio and the Multiplier and checks that the syntax of the variables is correct. The Evaluated Expression dialog is displayed showing the result of the rule using the values defined on the Variable Settings dialog.

Specifying Parameters

The columns in the NeoLynx Test Editor must be completed by the user, as they are dependent on the metabolites being analyzed and the internal standards used.

When a new Test Editor file is created the Mass fields and the IS, RRF and X fields are grayed out. The Mass fields default to 0.00 and the others to 1.00. When a Rule is selected for a test the fields required to perform the calculation for the rule will no longer be grayed out (See Figure 3.14 below). Values must be entered in these fields.

RULE	FUNCTION	IN CON	ΔX CON	MASS 1	MASS 2	MASS 3	MASS 4	MSG LOW	MSG HIGH	MSG OK	IS	RRF	X
2° Asc	1° Asc			3° Asc									
0:Absolute Intensity	No Function	0.00	0.00	0.0	0.0	0.0	0.0				1.00	1.00	1.00
1:2 Peak Ratio	No Function	0.00	0.00	0.0	0.0	0.0	0.0				1.00	1.00	1.00
2:4 Peak Ratio	No Function	0.00	0.00	0.0	0.0	0.0	0.0				1.00	1.00	1.00

Figure 3.14 Required Fields

To Add a Test

- Press the  toolbar button, select **Add Test** from the Test Editor **Edit** menu or right click anywhere in the table and select **Add Test** from the pop up menu displayed (Figure 3.15).

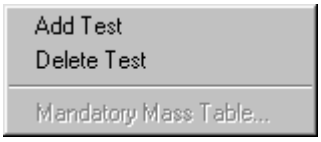


Figure 3.15 Pop up menu

- This will add a new blank line at the bottom of the table. Until all values for the test are correctly defined the number of the test is colored red and has a line through it (strikethrough) e.g.  .

- Enter data as required. See below for an explanation of the fields. When all relevant fields have been defined the test number turns gray and the strikethrough is removed.

The table should be saved by selecting **Save** or **Save As** from the Test Editor **File** menu.

A maximum of 255 tests can be defined in one grid.

To Modify a Test

1. Position the cursor on the field to be modified. This can be done by clicking on the field or by using the arrow keys.

   Enter data as required. See below for an explanation of the fields.

The table should then be saved by selecting Save or Save As from the Test Editor File menu.

To Delete a Test

2. Position the cursor on the test to be deleted. This can be done by clicking on any field in the row or by using the arrow keys.

3. Press the  toolbar button, select **Delete Test** from the Test Editor **Edit** menu or right click anywhere in the table and select **Delete Test** from the pop up menu displayed. This will delete the selected row.

Editing Data in a Cell

For the **Rule** and **Function** cells double click, on the cell and select a value from the drop down list box displayed. These values are defined in the Rule Definition and Function Name tables.

For all other fields to overwrite data in a cell click on the cell and type in a new value. If data for the same field type has been copied, by pressing the  toolbar button or **Copy** from the **Edit** menu, then the value can be pasted into the current cell by pressing the  toolbar button or selecting **Paste** from the **Edit** menu.

To edit data double click within a cell (e.g. to change C4-carnitine a to C4-carnitine b). All data in the cell is highlighted. Use the mouse or the keyboard arrow keys to position the cursor within the cell and change values as required.

Data from a single cell or multiple cells can be copied and pasted to other parts of the table. Areas may be selected with the mouse or the keyboard.

With the Mouse

To select	**Click with the left mouse button on**
A single cell	The required cell
A block of cells	The first cell in the block, hold down the mouse button and drag until the required cells are highlighted.
A row	The row number

With the keyboard

Use the arrow keys to position the cursor at the top left corner of the area to be selected, hold down the shift key and use the arrow keys to select an area.

When the required area has been selected press the  toolbar button or select **Copy** from the **Edit** menu. Move to the first cell of the paste area (using the arrow keys or by clicking on it) and press the  toolbar button or select **Paste** from the **Edit** menu. **Note**: The paste area must be of the same type as the copy area. i.e. if a Min Conc is copied it can only be pasted to another Min Conc cell.

Column Ordering Priorities

The tests within a test table may be ordered in ascending or descending order. The Function column MUST be ordered first, in ascending or descending function number order. Up to five more columns can be ordered within the function number ordering.

To select the ordering for a column, click on the row below the column header. This will set the priority in ascending alphabetical or numerical order and 1° Asc, 2° Asc etc will be displayed. Click again to change the ordering to a descending alphabetical or numerical order (1° Desc, 2° Desc etc will be displayed).

When a new Test file is created the default ordering parameters are:

Name The name of the test to be performed. Usually the name of a specific analyte, e.g. C3 carnitine. Test names must be unique, e.g. C4-carnitine a, C4-carnitine b, etc.

Rule The type of measurement to be performed. Three rules are already defined when a new table is created:

0:Absolute Intensity = use absolute peak intensities from the spectral data

1:2 Peak Ratio = determine the peak intensity ratio (Mass 1 / Mass 2)

2:4 Peak Ratio = determine the peak intensity ratio ((Mass 1 / Mass 2)/ (Mass 3 / Mass 4))

New ones can be added as required.

Function The number and description of the mass spectral function within the datafile to be processed. E.g. Parents of m/z 85 (acylcarnitines) and Neutral Loss of 102 Da (amino acids) may be functions 1:Acylcarnitines and 2:Neutral AA, respectively.

Min Conc The minimum concentration below which a message will be displayed.

Max Conc The maximum ratio or absolute peak intensity (Ion Ratio) above which a message will be displayed. Remember that the ratios will probably be small (2, 1, 0.2, etc.) and that absolute intensities will be quite high (e.g. $>10^4$).

Note: Max Conc must exceed Min Conc.

Mass1 The first mass of interest. This value must be greater than 20.

Mass2, 3, 4 When a new Test Editor file is created, the Mass fields are grayed out. When a Rule is selected for a test, the fields required to perform the calculation for the rule will no longer be grayed out. Values must be entered in these fields. This value must be greater than 20.

MsgLow Message written to the result column of reports if the absolute intensity or ratio falls below the **Min Conc** value.

MsgHigh Message written to the result column of reports if the absolute intensity or ratio lies above **Max Conc** value.

MsgOK Message written to the result column of reports if the absolute intensity or ratio lies between **Min** and **Max Conc** values.

IS The concentration of the Internal Standard. A value must be entered. The default is 1.

RRF The relative response factor, to be used if an internal standard is used that is not an analogue of the analyte of interest.

X A user definable variable that may be used in individual test calculations.

NeoLynx has in built checking routines, which verify certain criteria within a rule. If erroneous values are entered the number of the test turns red and has a line through it (strikethrough). An error message is also displayed as a tool-tip when the cursor is placed on the highlighted test number.

Processing

The **Processing** menu on the top line of the NeoLynx Test Editor allows access to the **Combine** and **Mass Measure** and **Peak Detection** parameters. The parameters defined here are stored with the individual NeoLynx Test Files and are unique to a test file.

Mass Measure

Full-scan MCA data is prepared by performing a single **Mass Measure** operation on the raw data. Mass measure allows the operator to perform mass measurement of a continuum (or MCA) spectrum in one command. Mass measure performs peak centering with optional background subtraction and/or smoothing.

The type of data acquired in a full-scan MCA experiment is shown in Figure 3.16 for constant neutral loss of 102 Da.

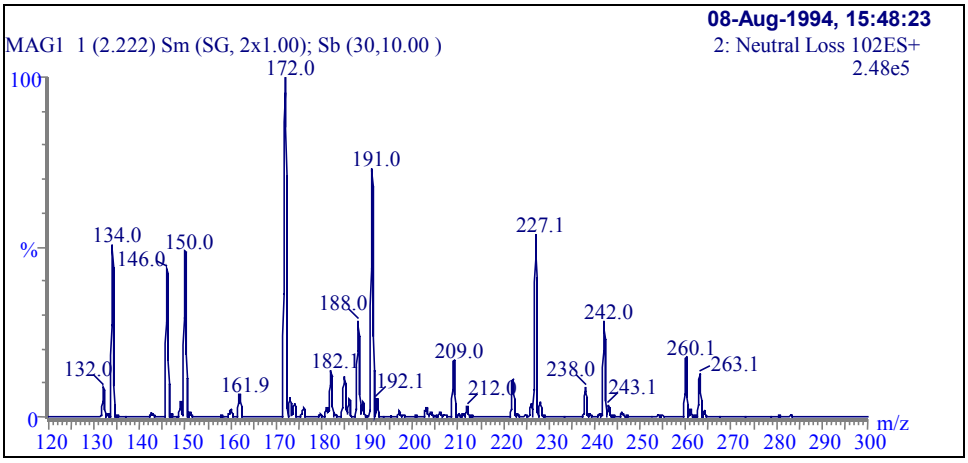


Figure 3.16 Constant Neutral Loss (102 Da) for an amino acid mixture after background subtraction and smoothing

The data shown in Figure 3.16 has been partially processed. In order for NeoLynx to be able to compare peak intensities, it is necessary to further process the data in order to generate a centroided spectrum as shown in Figure 3.17.

In Mass Measure, Background subtraction takes place if the **Background subtract** control is checked. The Mass Measure dialog gives access to the **Polynomial order** and **Below curve (%)** parameters which are described in the **Background Subtract** section of the MassLynx NT User Guide.

Mean smoothing takes place if the **Mean smooth** control is checked. The Mass Measure dialog gives access to the **Peak width**, **Number of smooths**, **Mean**, and **Savitzky Golay** parameters which are described in the **Smoothing** section of the MassLynx NT User Guide.

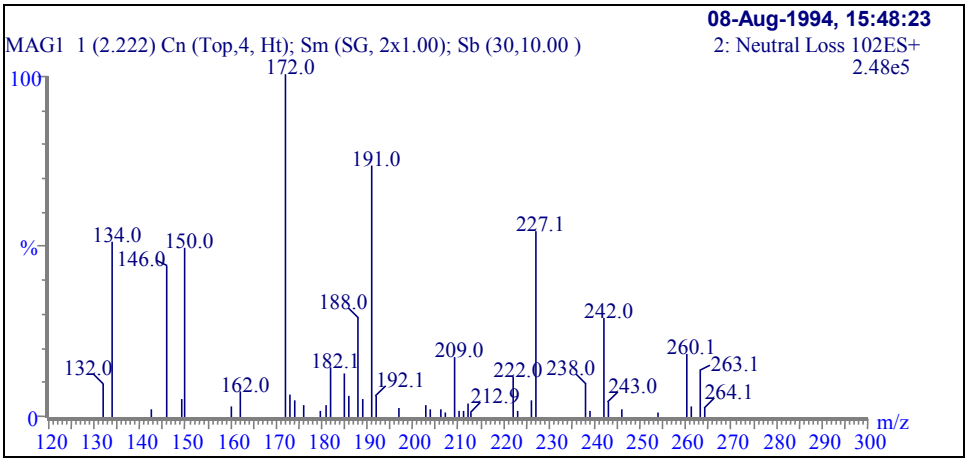


Figure 3.17 The same figure as shown In Figure 3.16 after centroiding

Peak Centering always takes place when the Mass Measure process is performed. The Mass Measure dialog gives access to the **Peak width at half height**, **Top**, and **Centroid top** parameters which are described in the **Center** section of the MassLynx NT User Guide.

The Mass Measure dialog box always retains the last set of parameters used.

When preparing data for analysis by NeoLynx, the Mass Measure parameters shown Figure 3.18 are recommended. To access the dialog press the  toolbar button or select **Mass Measure Parameters** from the **Processing** menu.

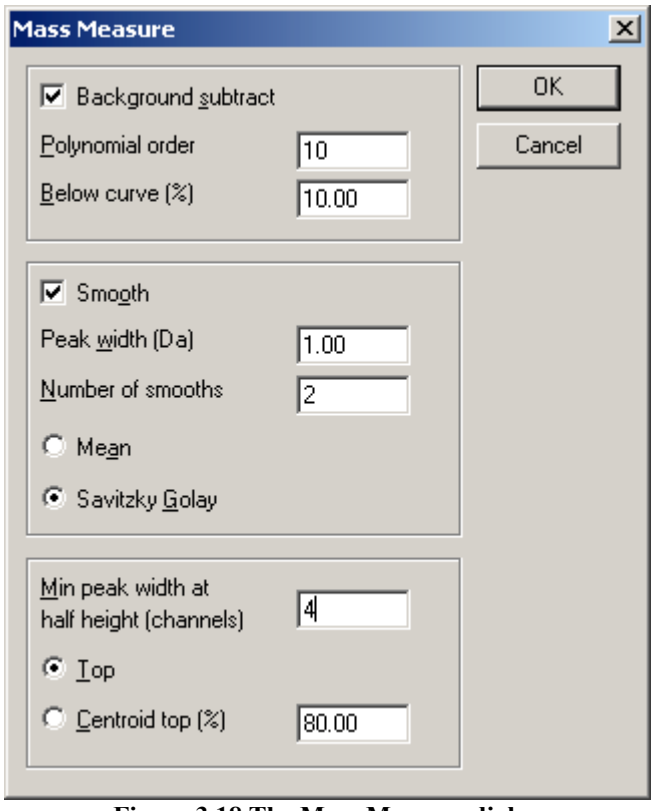


Figure 3.18 The Mass Measure dialog

Combine

For MRM and Continuum acquisitions, in order to prepare a spectrum that can be analyzed by the NeoLynx routine it is necessary to combine the spectra from across the chromatogram peak and to subtract any background noise that may appear on either side of the chromatographic peak.

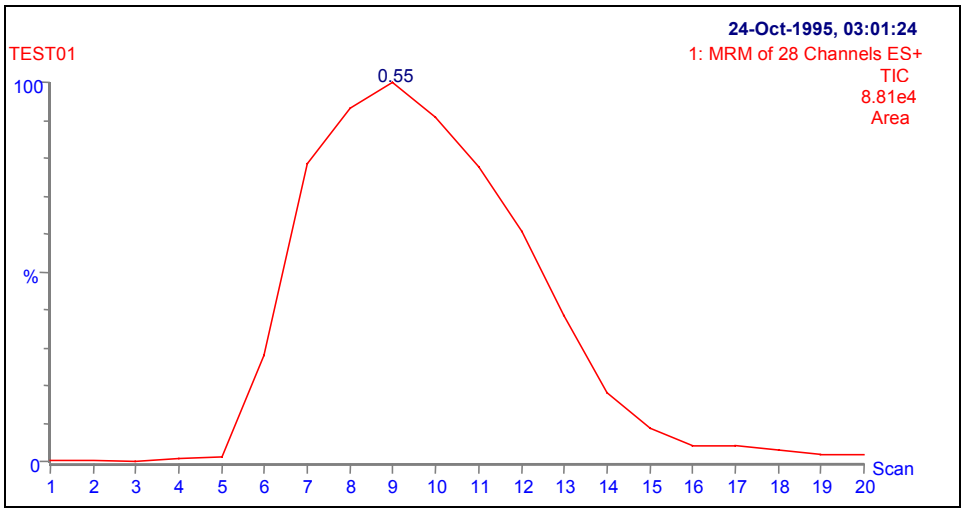


Figure 3.19 Total ion chromatogram for acylcarnitine MRM function (scan number abscissa)

The figure above displays the same data as Figure 2.14 but on a Scan rather than Time axis. To change the axis, select **View** from the **Chromatogram Display** menu and select the required **Axis Label**.

The **Combine Spectrum** dialog is shown in Figure 3.20. To access it press the  toolbar button or select **Combine Parameters** from the **Processing** menu.

Three **Scan ranges** are specified, and a **Background factor (X)**. The **Average** range contains the scans of interest across the chromatographic peak top. The **Subtract** ranges contain scans from the background of the chromatogram, on either side of the peak of interest. Typical values for the chromatogram shown in Figure 3.19 are shown in the dialog box Figure 3.20.

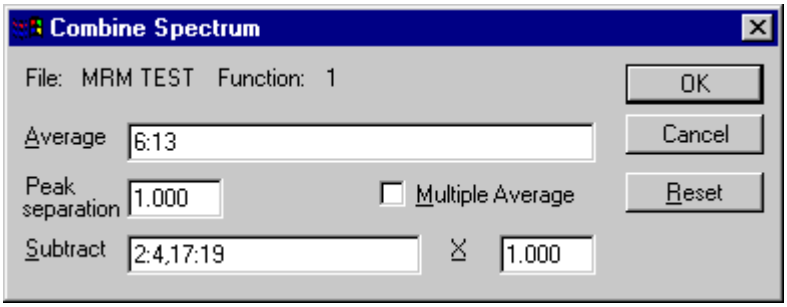


Figure 3.20 Combine spectrum dialog

Values can be entered by :-

- Typing values into the **Average** and **Subtract** boxes.
- Right clicking and dragging across a chromatogram peak at half height to enter the **Average** value, and then clicking and dragging either side of the peak to enter the **Subtract** ranges. The software will automatically enter these values in the correct boxes. **Note**: Ensure that the **Multiple Average** box is not checked as the first six ranges are automatically entered in the Average box and the next two in the Subtract box.

The scans across the peak top (scans 6 to 13) are averaged together, and from the result is subtracted the average of all the specified background scans (scans 2 to 4 and scans 17 to 19) multiplied by the **Background factor** (typically 1.000).

The **Peak separation** parameter is the spectral peak width in amu. For the MRM data acquired in this application, the peak separation should be set to 1.0.

A combined spectrum is shown in Figure 3.21and contains the ions of interest, as specified in the original scan function. The appearance of the data is similar to Figure 3.17

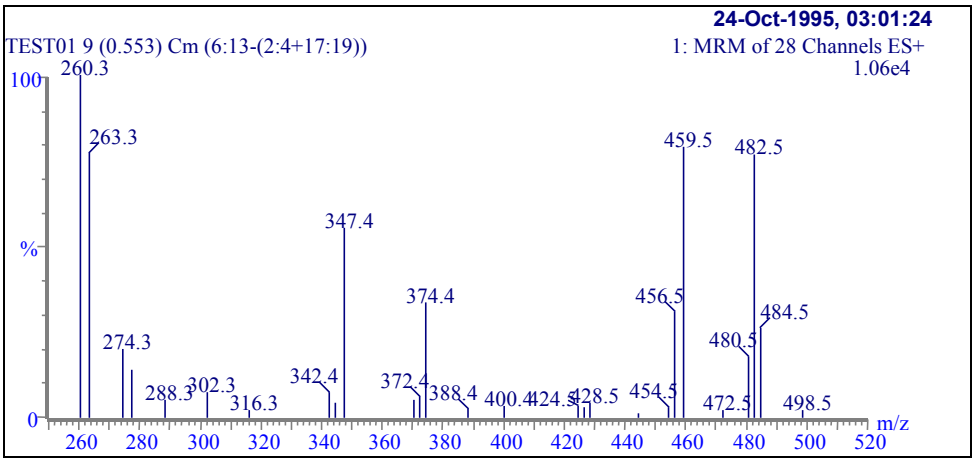


Figure 3.21 The combined spectrum derived from the acylcarnitine MRM function

Peak Detection

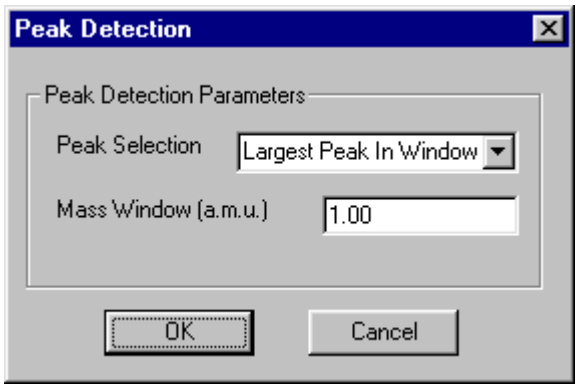


Figure 3.22 Peak Detection dialog

To access this dialog press the  toolbar button or select **Peak Detection Parameters** from the **Processing** menu.

Peak Selection The method by which NeoLynx selects the centroided peak of interest. The choices are:

- **Largest peak in window**: select the largest peak within the **Mass window** around the mass specified in the test editor.
- **Nearest to center**: select the nearest peak within the **Mass Window** to the mass specified in the test editor.

This option is included if the smoothing and peak centering parameters were incorrectly set. The peak of interest will most likely be the largest (and only) peak in the window. The default setting is for the largest peak in the window.

Mass window (a.m.u.) The mass window that is used to look for centered peaks around the specified mass of interest. In this application, a window of 1.0 Da will give ±0.5 Da around the target mass of interest.

Print Control

Printing of sample results reports is now controlled from the NeoLynx Browser.

The **Print Control** dialog allows access to the printing options available with the NeoLynx Test Editor. To access it press the  toolbar button or select **Print** from the **File** menu.

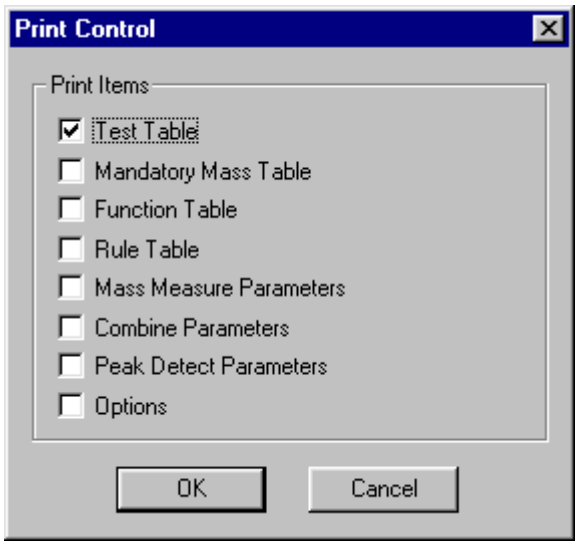


Figure 3.23 Print Control dialog

The report options dialog is used to control the type of reports sent to the printer. Check the relevant boxes to print the required information.

In order to fit the Test Editor Table on one page it is recommended that the default paper orientation is set to **Landscape** in the **Print Setup** dialog. To narrow columns so all fields are printed, position the cursor on the line between the column headings until ↔ appears, click and drag until the column is the required width. The appearance of the report may be viewed by selecting **Print Preview** from the **File** menu.

Chapter 4 NeoLynx Browser

Table of Contents

Figures

Overview

Processing of samples using the NeoLynx algorithm results in a NeoLynx Results file (*.nrf) being generated. This file is created in the root directory of the project in which the data are stored. The NeoLynx Results File is viewed using the NeoLynx Browser.

The NeoLynx Results files contain:

- All sample-related results
- All reduced mass spectral data used to generate the results
- All chromatographic data used to generate the results (if available)
- Complete NeoLynx Test File(s) used during the processing
- Sample-related information

The title bar of the NeoLynx Browser displays the name of current NeoLynx Results File (*.nrf) and the name of the currently active NeoLynx Report Scheme (*.nrs). The Report Scheme controls the type of printed and electronic reports generated.

Accessing the NeoLynx Browser

To access the NeoLynx Browser select **NeoLynx, Browser** from the MassLynx Shortcut Bar

The NeoLynx Browser Screen

The Browser Screen (Figure 4.1) is split into 7 panes. The Chromatogram Pane may not be displayed, depending on the type of data acquired.

- The Plate Paneshowing the plate layout and tested wells.
- The Results Table Paneshowing a list of detected compounds.
- The Function Pane showing a list of the functions used to acquire data.
- The Results Summary Pane showing the abnormal messages or Pass Message.
- The Sample Description Pane showing sample information for the currently selected vial.
- The Spectrum Pane showing the spectrum for the vial and test selected.
- The Chromatogram Pane showing the chromatogram for the vial and test selected. Note this is not shown if chromatogram data are not available.

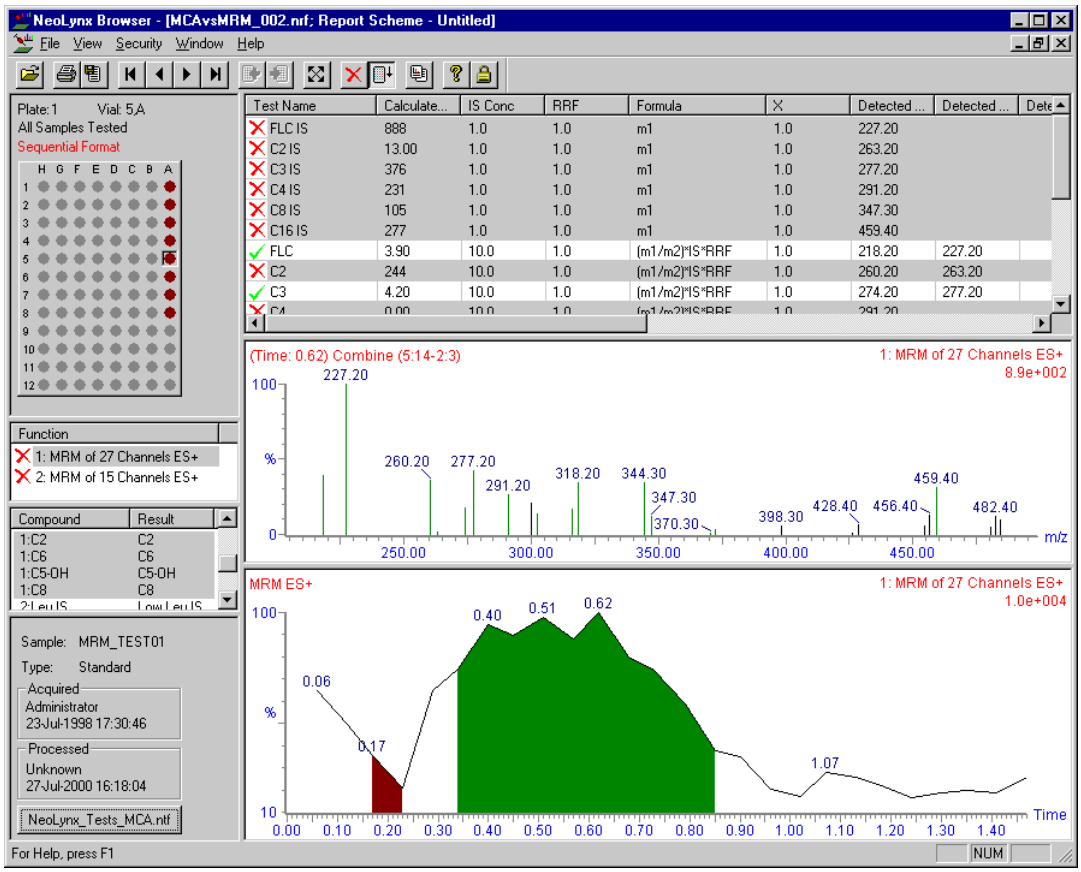


Figure 4.1 The NeoLynx Browser

The NeoLynx Browser Toolbar

Toolbar button	Menu equivalent	Purpose
	File… Open	Open an existing NeoLynx Browser report
	File… Print	Print the results from a NeoLynx Browser file using the specified NeoLynx Report Scheme
	File… Save Electronic Reports	Generate electronic reports using the specified NeoLynx Report Scheme
		NeoLynx Browser go to first Vial
		NeoLynx Browser go to previous Vial
		NeoLynx Browser go to next Vial
		NeoLynx Browser go to last Vial
		Display previous plate

		Display next plate
		Display default range
		Display Fails Only
		Display Samples sequentially
	File… Report Scheme Settings	Invoke Edit Report Scheme Settings dialog
	Help… About NeoLynx Results Browser	Display program information, version number and copyright

Getting Started

To Open an Existing NeoLynx Browser File

1. Press the  Toolbar button, or select **Open** from the **File** menu.
2. Select the required NeoLynx Browser results file (*.nrf) and press **Open**.

View Options

A number of options are available to change the appearance of the Browser screen, to access them select **Options** from the **View** menu.

The Spectrum Page

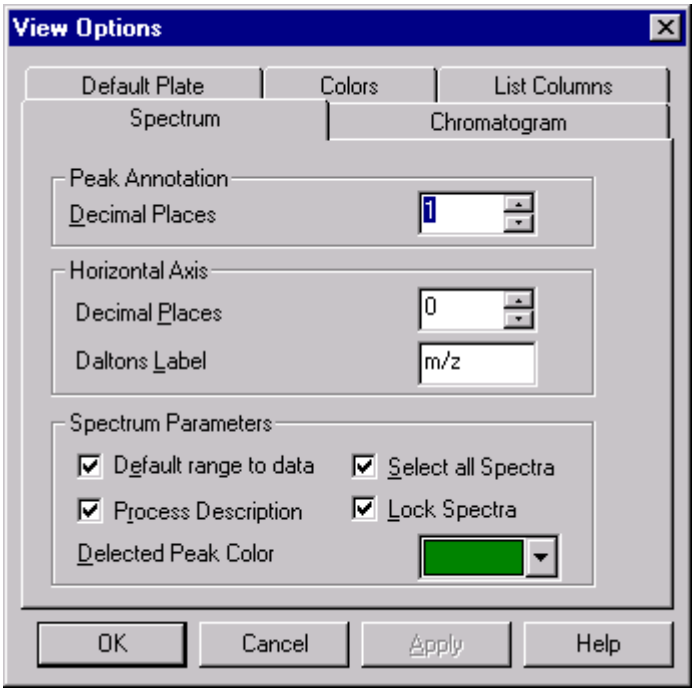


Figure 4.2 The Spectrum Page

Peak Annotation Decimal Places	Change this value, by pressing the arrows, to change the number of decimal places displayed on peak annotation. Range 0 to 4.
Horizontal Axis Decimal Places	Change this value, by pressing the arrows, to change the number of decimal places displayed on the horizontal axis. Range 0 to 4.
Daltons Label	Type in the text to appear as the label for the horizontal axis, for mass spectral data. Maximum length is 8 characters.
Default range to data	When checked the default mass display range of a spectrum will be based upon the peaks the spectrum contains, only the mass range, which actually contains data, will be displayed. If unchecked the default display range will be the actual mass range the spectrum was originally acquired over.
Process description	Check this box to display the Process Description in the top left corner of the Spectrum. This describes the processing used to derive the displayed spectrum from the raw data.
Select all Spectra	If this box is checked then when a new vial is selected all functions are selected in the Functions pane and all spectra for the vial are displayed. Individual spectra can be displayed by clicking on the test in the Function or Test Results panes.
Lock spectra	If this box is checked, the spectra will be locked to the mass and intensity defined for the individual functions in the NeoLynx Test File. In such a situation, the nominated peak(s) will be set to the pre-defined intensity on the display, and all other peaks will be scale accordingly - be aware that some peaks may be sturated in this display mode.
Detected peak colour	Allows for selection of the colour of the peaks in the spectrum display that have been used in the NeoLynx calculations.

The Chromatogram Page

Peak Annotation Decimal Places	Change this value, by pressing the arrows, to change the number of decimal places displayed on peak annotation.
Horizontal Axis Decimal Places	Change this value, by pressing the arrows, to change the number of decimal places displayed on the horizontal axis.
Label	Type in the text to appear as the label for the horizontal axis.
Show Combine Parameters	Check this box to display the combine parameters (Spectrum Average and Background Subtract) used to generate the Spectrum in the Spectrum pane. The Combine parameters are also displayed on the chromatogram (green = average, red = background subtract). The colors used to display the combine and background subtract regions are user-selectable.

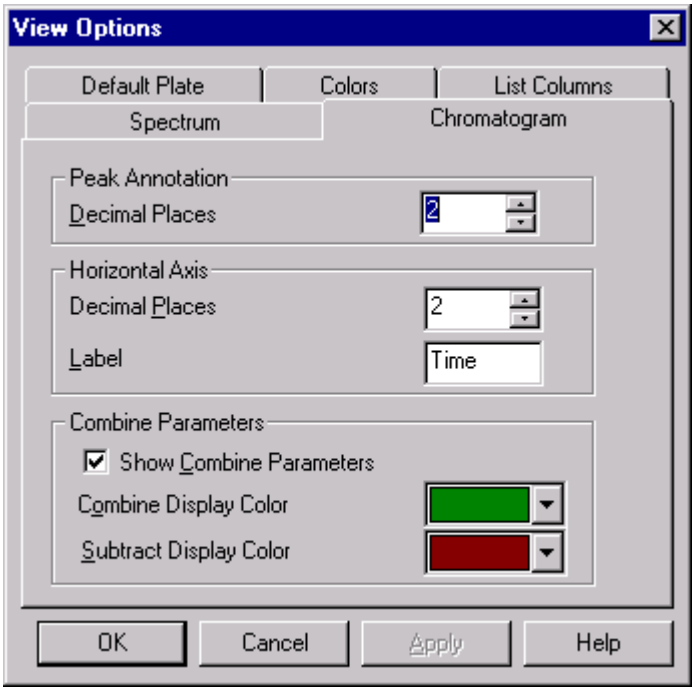


Figure 4.3 The Chromatogram Page

The Default Plate Page

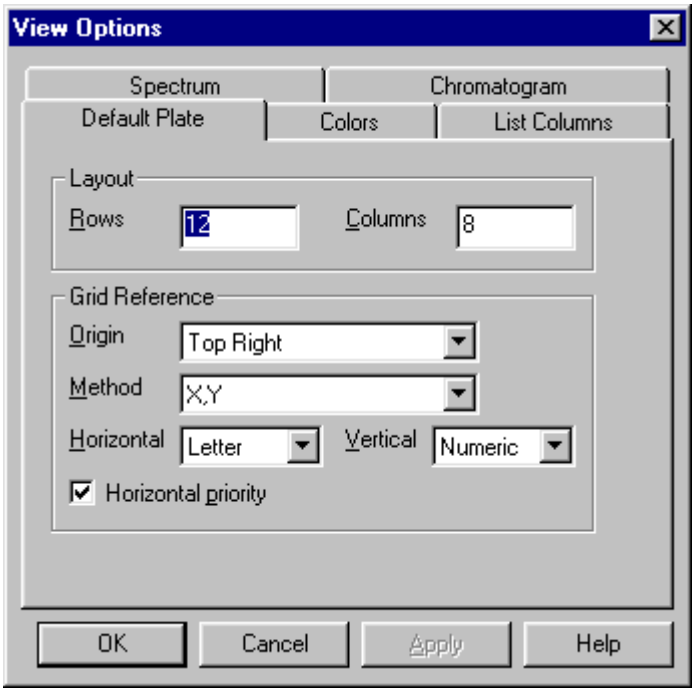


Figure 4.4 The Default Plate Page

Rows Enter the number of rows on the plate to be used. Range 1 to 32.

Columns Enter the number of columns on the plate to be used. Range 1 to 32.

Origin This is the corner of the rack that the vial grid referencing starts from. Select one of Top Right, Top Left, Bottom Right or Bottom Left from the drop down list box.

Method This is the method of numbering the vials on a plate. There are three options

- XY which references the vials A1, B1 etc.
- Sequential Discontinuous which numbers the vials 1, 2, 3 across a row, left to right if origin is top left, and then starts the next row from the left again.
- Sequential Continuous which numbers the vials 1, 2, 3 across a row, left to right if origin is top left, then continues number the next row, right to left etc.

If a Gilson autosampler or a Waters 2700 autosampler is used with NeoLynx then the vial referencing must be set to either sequential continuous or sequential discontinuous.

Horizontal If the method chosen is X,Y then this box becomes enabled. It allows horizontal referencing of the plate to be a number or a letter.

Vertical If the method chosen is X,Y then this box becomes enabled. It allows vertical referencing of the plate to be a number or a letter.

Horizontal Priority Check this box if samples are to be acquired horizontally across the plate.

If Referencing = X,Y, Horizontal = Letter, Vertical = Number and Horizontal Priority is checked, this will result in samples being acquired in the order A1, A2, A3. If the Horizontal Priority box is not checked samples will be acquired in the order 1A, 1B, 1C etc.

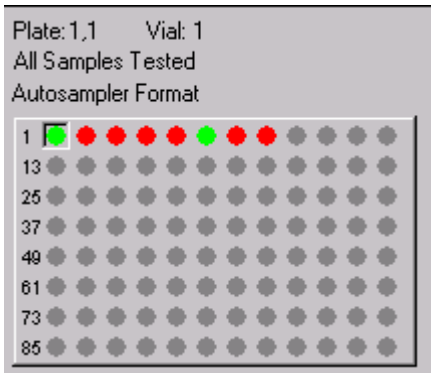
If Referencing = sequential continuous or discontinuous and Horizontal Priority is checked, this will result in samples being acquired from row 1 then row 2. If the Horizontal Priority box is not checked, samples will be acquired from column 1 then column 2 etc.

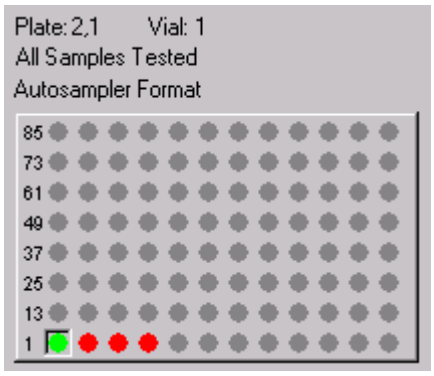
Plate Display

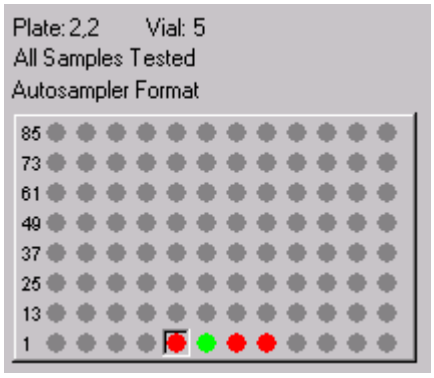
For autosamplers controlled by the MassLynx software a plate layout will have been defined (see the MassLynx Guide to Data Acquisition). This information is stored in the raw data header file and cannot be changed. i.e. When the browser report is opened the plate will be displayed in the format defined for the acquisition and not the one defined on the Browser Default Plate page.

For autosamplers not controlled by the MassLynx software, this page is used to organize the data acquired so that it can be displayed in plate format.

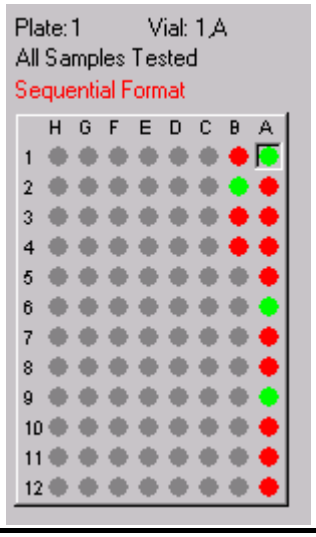
Sample information can be displayed in the format it was acquired or sequentially. i.e. If data for 16 samples has been acquired on three different plates as shown below, it can be viewed on the three separate plates by pressing the  and  (previous and next plate) toolbar buttons.







Pressing the  (sequential samples) button will display the data sequentially in the format defined on the Default Plate page. This type of display is useful for examining incomplete runs (Sample(s) not tested) to determine the sequence number of the missed sample.



Sequential Format is displayed above the plate to indicate that the plate is displayed in the sequential sample format.

Pressing the  button a second time will return to the previous format.

The Colors Page

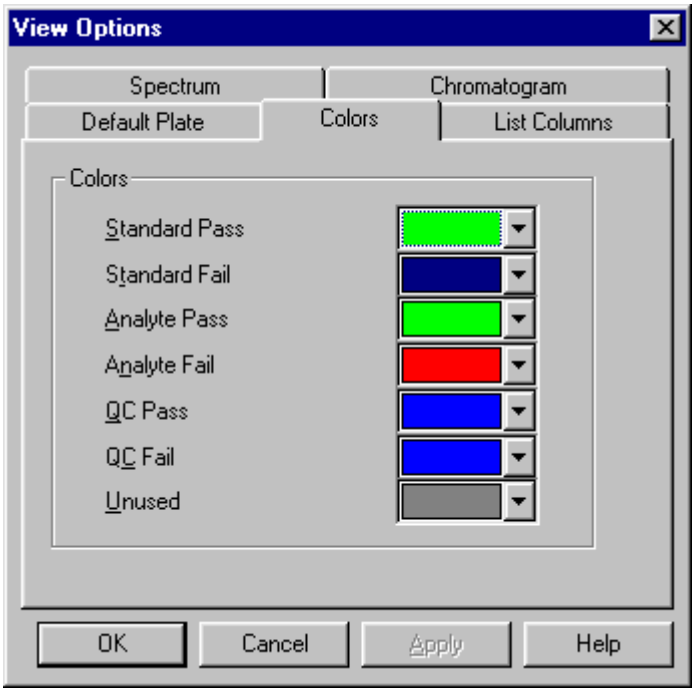


Figure 4.5 The Colors Page

This page allows the user to define the colors used on the Plate pane. Select a color from the drop down list box.

Standard Pass The sample was defined as Standard in the Sample List and passed all tests defined in the NeoLynx Test File.

Standard Fail The sample was defined as Standard in the Sample List and failed one or more tests defined in the NeoLynx Test File.

Analyte Pass The sample was defined as Analyte or Blank (or left blank) in the Sample List and passed all tests defined in the NeoLynx Test File.

Analyte Fail The sample was defined as Analyte or Blank (or left blank) in the Sample List and failed one or more tests defined in the NeoLynx Test File.

QC Pass The sample was defined as QC in the Sample List and passed all tests defined in the NeoLynx Test File.

QC Fail The sample was defined as QC in the Sample List and failed one or more tests defined in the NeoLynx Test File.

Unused There was no sample defined for this position on the plate.

The List Columns Page

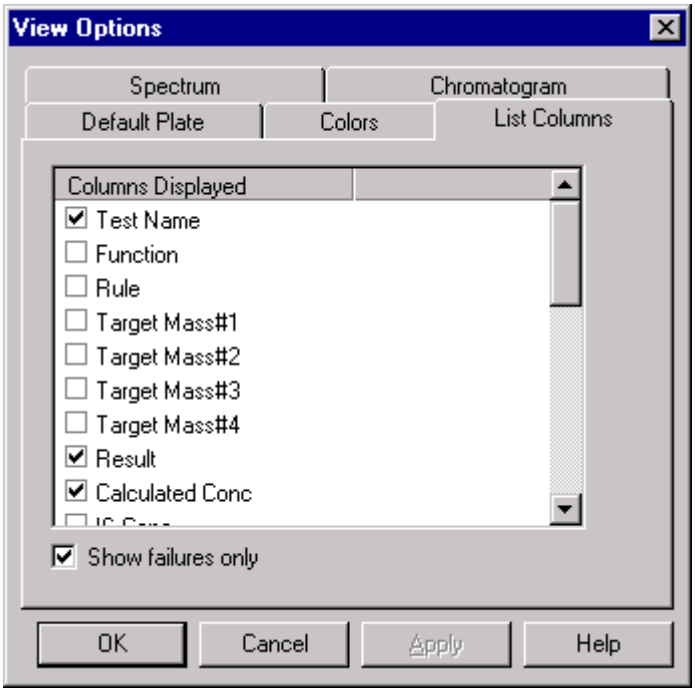


Figure 4.6 The List Columns Page

This page allows the user to define which information is displayed in the Results Table pane.

Check the boxes next to the fields required.

Columns can be removed from the display by right clicking on a column and selecting the **Remove Column** pop-up menu, which appears. Columns can only be restored from the List Columns page.

Show failures only — Check this box to show only failed tests. Pressing the  toolbar button will also perform this action, pressing the button a second time will display all results again

Function — The number and name of the function defined in the Test.

Rule — The number and name of the Rule that was applied in the Test. E.g. 1:2 Peak Ratio = determine the peak intensity ratio (Mass 1 / Mass 2).

Target Mass 1-4 — The Target Mass(es) defined in the Test. These will be 0.00 if not used for the rule.

Result — The message defined in the Msg Low, Msg High or Msg OK field for the Test, depending on th e result of the test.

Calculated Conc — The Calculated Concentration defined in the Test.

IS Conc — The concentration of the internal standard defined in the Test.

RRF — The Relative Response Factor defined in the Test.

Formula — The complete formula (Ion Ratio * Multiplier) defined in the Test.

X	The value of the X variable defined in the Test.
Detected Mass 1-4	The masses detected and used in the calculations.
High Conc	The Max Conc defined in the Test.
Low Conc	The Min Conc defined in the Test.
Units	The units defined in the Test.
Spare 1-5	The values entered in the Spare 1-5 columns in the Sample List.

Other Display Options

To Display Two Documents Simultaneously

1. Select **Open** from the **File** menu, this will display a new window over the top of the previous one.

2. Select **Tile** or **Cascade** from the window menu. This will display the two child windows in the style chosen.

3. Repeat the above steps as often as required.

To Display Two Different Views Of The Same Document

Select **New Window** from the **Window** menu. A copy of the current window created, named "*filename*:2". Select **Cascade**, **Tile Horizontally** or **Tile Vertically** from the **Window** menu to display this new window along side the existing document to compare results of similar searches.

To Change the Size of a Pane

To change the size of the panes on display position the mouse pointer on the line between the two panes until a  or  symbol appears. Click and drag until the pane is the required size.

To Change the Size of the Display

On a standard screen set to 800 by 600 pixels there may not be enough room to display all the information in all of the panes. Changing the desktop settings will display more information.

1. Press the **Start** button, select **Settings** and then **Control Panel**.

2. From the Control Panel select **Display**.

3. On the **Settings** page change the **Desktop Area** to 1024 by 768 pixels or above.

4. Press the **Test** button and if the test page displays correctly press the **OK** button.

Other Menu Options

File Menu

Load on startup If this option is selected, from the **File** menu, the next time the Browser is opened then the last Browser file viewed will be automatically loaded. A tick mark appears next to the item when selected, selecting the option again will turn it off.

Send To If this option is selected then an e-mail message is created with the currently selected report attached to it. Enter the address to send it to and press the send button.

To view the report, the person receiving the e-mail must have MassLynx (with the NeoLynx option) or a stand-alone copy of the NeoLynx Browser installed. The attachment can then be saved as normal and then opened in the NeoLynx Browser. It can also be opened by double clicking on the report in the e-mail message.

View Menu

Refresh This option rereads the current Browser Report and updates the display information. This command should be used if the content of the Browser Report has changed as a result of processing further samples. Pressing the **F5** function also performs this action.

Toolbar If this option is selected, from the **View** menu, then the Toolbar will be visible. A tick mark appears next to the item when selected. Selecting the option again will turn it off.

Status Bar If this option is selected, from the **View** menu, then the Status Bar will be visible. A tick mark appears next to the item when selected. Selecting the option again will turn it off.

Window Menu

New Window Selecting this item will create a copy of the current window. This is useful for displaying different views of the same document. The second document will have a ':2' after the name. To change between documents select the document required from the documents listed at the bottom of the Window menu. A tick will appear next to the document, which is currently active.

Cascade Arranges document windows so that the title bar of each window is visible.

Tile Arranges open windows side by side on the screen, dividing the available space equally between the open windows so that all of them are visible.

Arrange icons Arranges all iconized windows into rows.

The Plate Pane

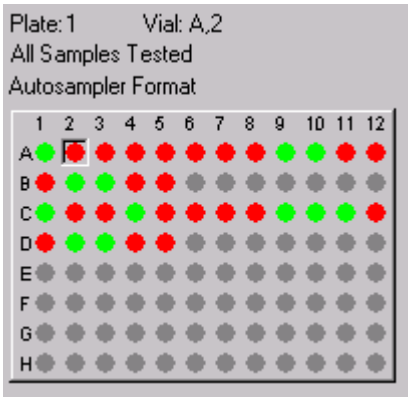


Figure 4.7 The Plate Pane

The Plate Pane displays information about all the samples from the currently selected plate. The vials are color coded to indicate whether they contained a sample, which passed all the tests or failed one or more tests defined in the NeoLynx Test File. Different colors can be assigned to the different Sample Types defined in the Sample List.

All Samples Tested is displayed if all the samples submitted for analysis have been processed. If samples have not been tested, the message **Sample(s) not tested** is displayed.

These colors can be changed see The Colors Page on page 4-11.

The currently selected vial is shown recessed. Change the current vial by clicking on a new vial, using the vial selection toolbar buttons (, , , and) or arrow keys.

Clicking on a vial, will display detailed information about that vial in the other panes of the screen.

The current plate number can be changed by using the previous plate and next plate toolbar buttons.

The appearance of a plate can be modified using the View Options command. This enables the number of rows and columns, reference labels and vial colors to be set. View Options on page 4-7 for more details.

Pressing the (sequential samples) button will display the data sequentially in the format defined on the Default Plate page. See Plate Display on page 4-10 for more details.

The Results Table Pane

Test Name	Low Conc	High Conc	Result	Calculated Conc	Units
✓ Phe Intensity	2.00e+4	1.00e+6	OK	5.05e+4	Counts
✗ Phe	0.00	200	High Phe	258	umoles/L
✓ Tyr	0.00	400	OK	77.50	umoles/L
✗ Phe/Tyr	0.00	2.50	High Phe/Tyr	3.33	-

Figure 4.8 The Results Table Pane

This pane is used to display information about the Tests performed. How to display a field and the field descriptions are described fully in The List Columns Page on page 4-13.

By default when a well is selected all results are displayed. To display only the failed tests press the  toolbar button, press the button a second time to return to displaying all results. Failed tests are highlighted as in the example above.

Columns can be removed from the display by right clicking on a column and selecting the **Remove Column** pop-up menu that appears. Columns can only be restored from the List Columns page

The width of the columns can be changed, by positioning the mouse pointer on the heading between two columns until the ✣ symbol appears, and then clicking and dragging until the column is the required width.

The order in which the columns are displayed can be changed. Click on the column heading, hold the button down and drag the column header to the required position.

The Function Pane

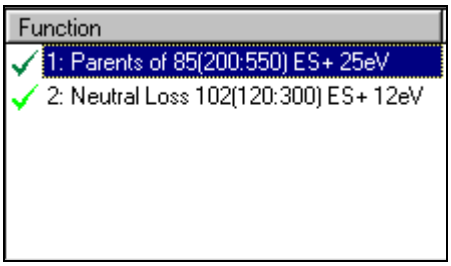


Figure 4.9 The Function Pane

This pane shows a list of the functions used to acquire data. The description of the function displayed in this pane is the MassLynx Scan Functions Editor definition, not the description used in the NeoLynx Test File.

If all the Tests for this function, defined in the NeoLynx Test File, have been passed a green tick appears next to the function, otherwise a red cross is displayed.

Click on a function to display the results for this function in the Results Table pane, the Spectrum Pane and the Chromatogram Pane, if active. Multiple entries can be selected by holding **Ctrl** key and clicking on the required entries or holding down the **Shift** key and clicking on the last entry in a block. Selection of multiple functions will update the Spectrum, Chromatogram and Results Pane with the selected functions.

The Results Summary Pane

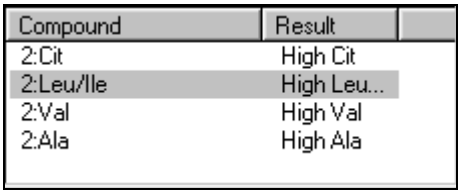


Figure 4.10 The Results Summary Pane

If any test has failed this pane shows the message defined in the MSG HIGH or MSG LOW fields in the NeoLynx Test file. The Result highlighted corresponds to the entry selected in the Results Table pane.

If no tests have failed the Pass Message defined in the NeoLynx Test File is displayed (**NeoLynx Test File Editor**, **Processing**, **Options, Process Options**).

The Sample Description Pane

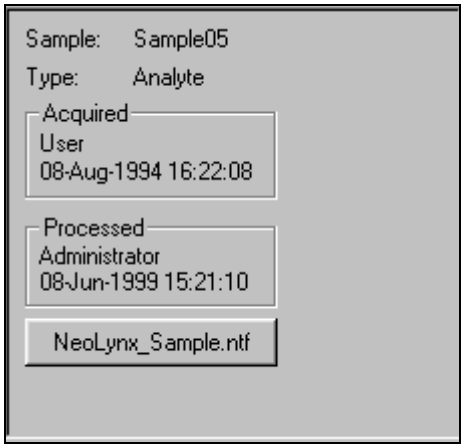


Figure 4.11 The Sample Description Pane

This pane shows the description of the sample for the well selected in the plate pane.

Sample The name of the Sample defined in the Sample List.

Type The Sample Type defined in the Sample List.

Acquired The name of the person logged on to MassLynx at the time of data acquisition and the time of acquisition.

Processed The name of the person logged on to MassLynx at the time of data processing and the time of processing.

The name of the NeoLynx Test File used to process the data is shown on the button at the bottom of the pane. Pressing this button will display the relevant NeoLynx Test File in read-only mode.

When a NeoLynx Results File is created, the NeoLynx Test File used to process the acquired data is stored as an integral part of it. The file displayed by pressing the button on this pane is the saved file and so any changes made to the NeoLynx Test File after the Results File was created will not be displayed.

The Spectrum Pane

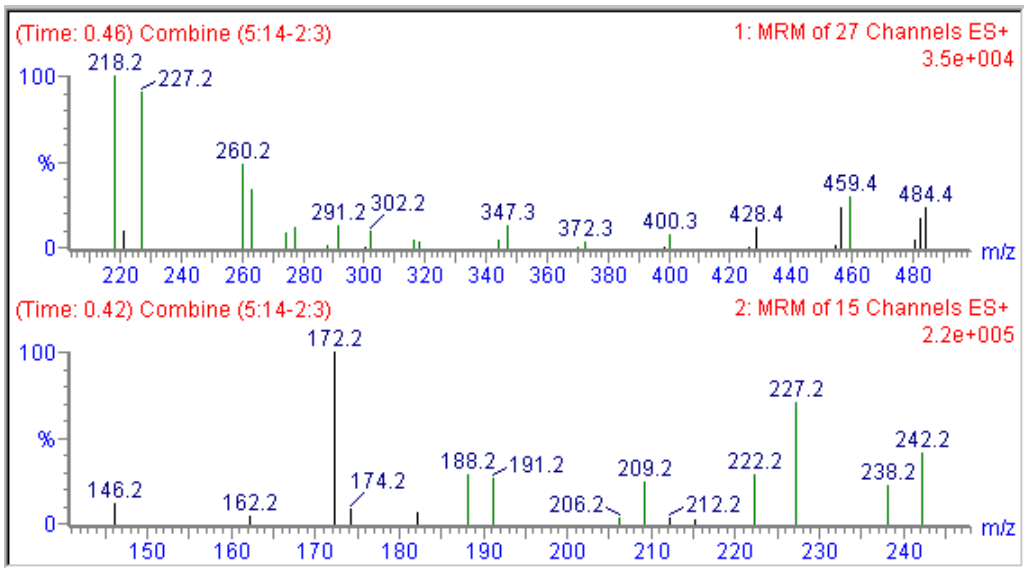


Figure 4.12 The Spectrum Pane

This pane displays, the mass Spectra of the entry (or entries) highlighted in the Function pane, the Results Table Pane or the Results Summary pane.

Peaks that match the detected peaks in the Results Table pane are displayed in green.

The Spectrum Pane will appear blank if an unused vial is selected. If the pane is not large enough to display all the selected spectra, a scroll bar automatically appears on the right of the pane.

The text in red on the left of the pane shows the mass spectrum retention time. The process description will also be displayed if the relevant box is checked on the **View, Options, Spectrum page.**

The text on the right of the pane shows the acquisition function type and ionization mode followed by the absolute intensity of the largest peak in the spectrum, on the next line.

Altering the range of the horizontal axis (zoom) with the mouse

Click with the mouse at one end of the region of interest, and without releasing the button, drag the mouse horizontally to the other end. As the mouseis dragged a "rubber band" is stretched out to indicate the range selected; don't go beyond the bounds of the axis. When the mouse button is released the selected range will be re-displayed to fill the current window.

This operation can be repeated as often as required.

Pressing the  toolbar button will restore both the Spectrum and Chromatogram display to the default range.

Viewing other spectra

Each entry can have one or more spectra associated with it. To view another spectrum page down using either the scroll bar or the arrow keys.

The Chromatogram Pane

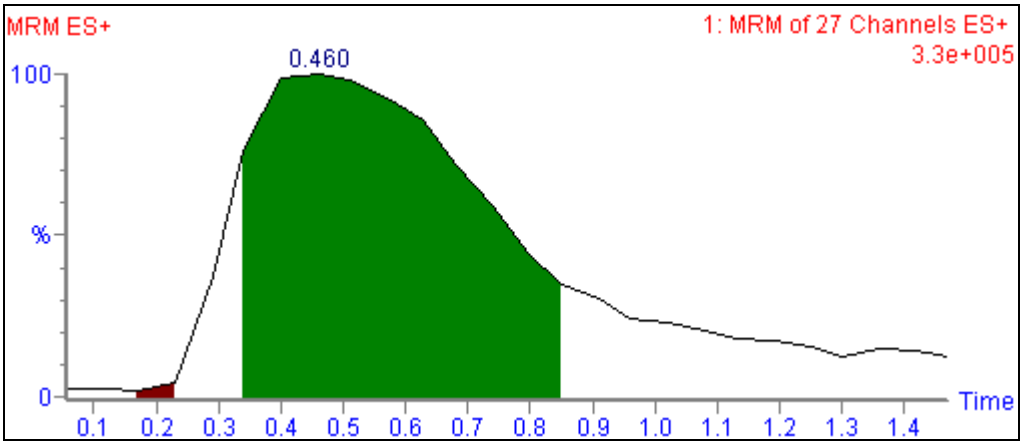


Figure 4.13 The Chromatogram Pane

This pane displays the processed chromatogram of the entry highlighted in the Results Table pane. If chromatogram data is available, the chromatogram pane is always displayed beneath the spectrum pane. If a chromatogram is expected, but not displayed, the NeoLynx Browser may have minimized the chromatogram window. Position the cursor at the bottom of the spectrum pane until it changes to  . Click and drag upwards until the chromatogram pane is the required size.

The part of the chromatogram peak used for the combine process is displayed in color if **Show Combine Parameters** is checked on the **View, Options, Chromatogram page**.

Each peak is annotated with the retention time. The text in red on the left of the pane is the chromatogram description.

The text on the right of the pane is an indication of the maximum intensity of the chromatogram.

Altering the Range of the Horizontal Axis (Zoom) with the Mouse

Lick on one end of the region of interest, and without releasing the button, drag the mouse horizontally to the other end. As the mouse is dragged, a "rubber band" is stretched out to indicate the range selected; don't go beyond the bounds of the axis. When the mouse button is released, the selected range will be re-displayed to fill the current window.

This operation can be repeated as often as required.

Pressing the  toolbar button will restore both the Spectrum and Chromatogram display to the default range.

Viewing Other Chromatograms

Each entry can have one or more chromatograms associated with it. To view another chromatogram page down using either the scroll bar or the arrow keys.

Printing and Reporting

A variety of printed and electronic reports can be produced using the NeoLynx Browser. Formatting of the outputs is described in this section, and examples of the supplied electronic reports are shown in Appendix E.

To Print NeoLynx Results

Press the  Toolbar button, or select **Print** from the **File** menu. This will display the Print control dialog.

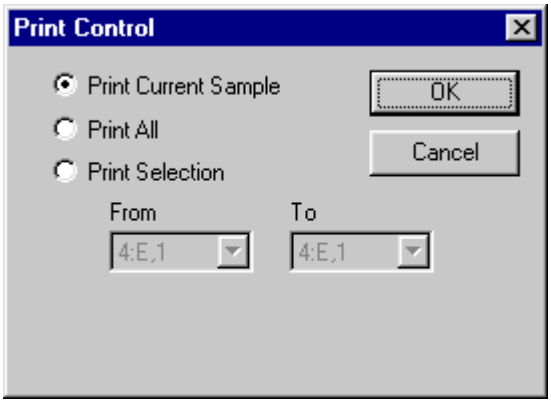


Figure 4.14 The Print Control dialog

Select **Print Current Sample** to print information for the currently selected vial.

Select **Print All** to print information for all vials on all plates.

Select **Print Selection** and select a **From** and **To** vial number from the drop down list boxes.

Press **OK** to print the report.

The format of the reports is defined by the selected NeoLynx Report Scheme, which is covered in detail later in this section.

To Generate NeoLynx Electronic Reports

Press the  toolbar button or select **File**, **Save Electronic Reports** from the menu. This will display the advice dialog (Figure 4.15).

The Report Settings displayed will depend on the options selected in the NeoLynx Report Scheme. Pressing **OK** will result in the electronic reports being generated.

This message is not shown when electronic reports are generated automatically from a Sample List – Run Scheme after batch is checked in the NeoLynx Test File, Print Options.

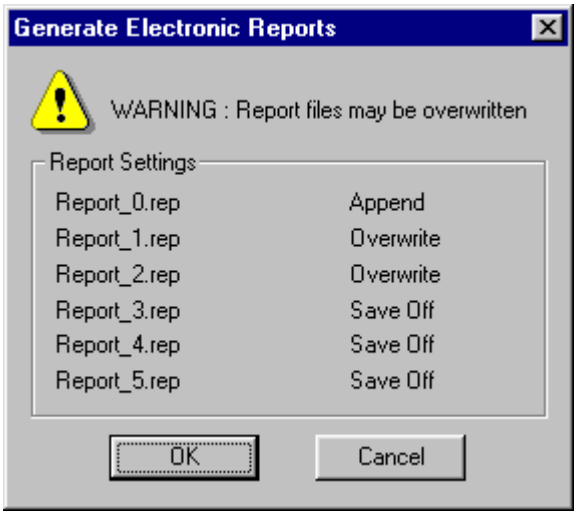


Figure 4.15 Generate Electronic Reports: advice dialog

If the current version of a report is open (e.g. being viewed in Notepad or Excel) then the file will not be updated or overwritten. All closed files will be updated.

Report Schemes

Access to NeoLynx Report Schemes is controlled by the MassLynx Security program. Schemes may not be edited and saved unless accessed by an authorized operator. If unauthorized access is attempted, the following message will be generated:

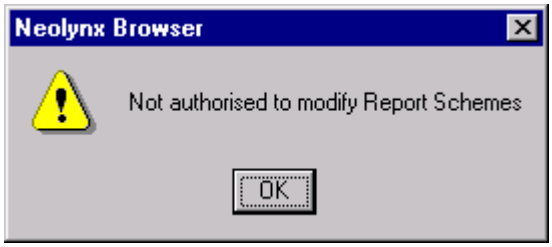


Figure 4.16 Warning dialog

To Open a Report Scheme

1. Press the  toolbar button or select **Report Scheme Open** from the file menu.
2. Select the required *.nrs file from the dialog displayed.
3. Press **Open**.

To Save a Report Scheme

1. Select **Report Scheme Save As** from the file menu.
2. Select the required location and enter a *.nrs filename in the dialog displayed.
3. Press **Save**.

Report Scheme Settings

The information on printed reports can be defined via the Report Scheme Settings dialog. To access the dialog select **File, Report Scheme Settings**.

The examples shown below, use the NeoLynx.nrs report scheme supplied with the NeoLynx software.

The Report Control Page

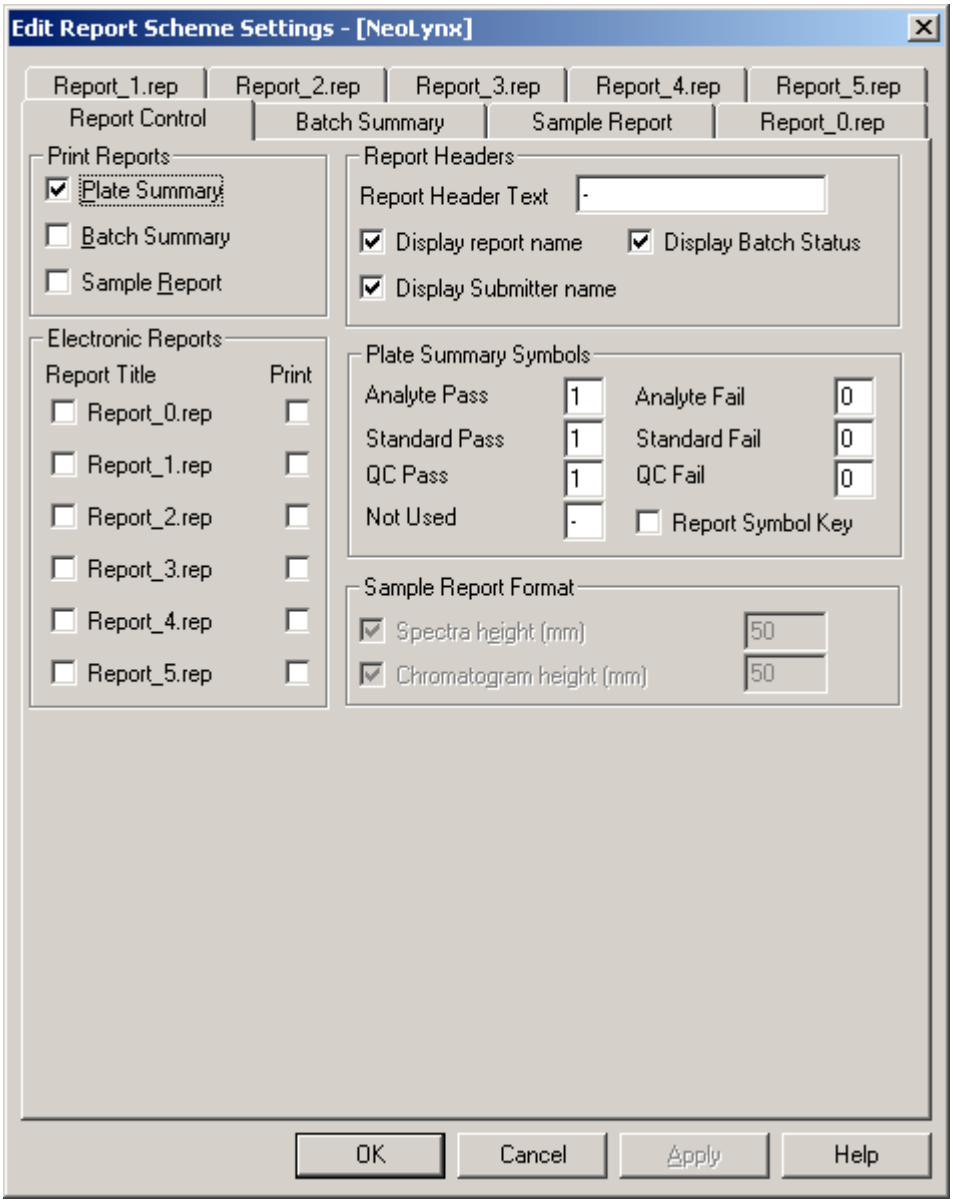


Figure 4.17 The Edit Report Settings dialog, Report Control Page

Print Reports

Check the **Plate Summary** box to produce a Plate report. This is a picture of the plate with the symbols defined in the **Plate Summary Symbols** representing Passed, Failed and Unused wells. Different symbols can be used for Standard, QC and Analyte samples. See below for details, and Figure 4.19 for an example.

Check the **Batch Summary** box to produce a summary report for a Batch of Samples. **Note** the number of samples printed is controlled from the Print Control dialog (To Print NeoLynx Results on page 4-21). For details on the content of this report see The Batch Summary Page on page 4-24.

Check the **Sample Report** box to produce a more detailed report for a sample.

Electronic Reports

Reports can be saved in an electronic format and/or printed. Check the **Report Title** boxes for the required reports and check the corresponding **Print** box to print a copy.

Report Headings

Enter text to appear at the top of a report in the **Report Header Text** field. This text will be displayed between the Report name and Submitter name if selected, see below.

Check the **Display report name** box to display "NEOLYNX <*report type*> REPORT" at the top of the report. If this text is not required make sure the box is unchecked and enter your own text in the Report Header Text field described above.

Check the **Display Submitter name** box to display the name of the submitter. the submitter is the name of the person logged on to MassLynx at the time of data acquisition.

Check the **Display Batch Status** box to display either "All Samples Tested" or "Samples Not Tested" in the upper right field to advise of the processing status.

Plate Summary Symbols

Enter a character into each field to be displayed on the Plate Summary of the printed report.

Check the **Report Symbol Key** box to print a list of symbol definitions on the report.

Sample Report Format

To print the spectra associated with a sample check the **Spectra height (mm)** box and enter the height required.

To print the chromatograms associated with a sample check the **Chromatogram height (mm)** box and enter the height required.

The Batch Summary Page

A Batch Summary report is a tabulated list of all samples that have been processed. Samples are grouped in sets of 96 (to reflect microtitre plate formats) and represent a simple summary of the samples analyzed. Information may be configured at the top of the Batch Report (Batch Summary header), and the columns in the main body of the report (Summary Data Fields). An example is shown in Figure 4.20**.**

In a Batch Summary 'failed' samples are highlighted in bold type.

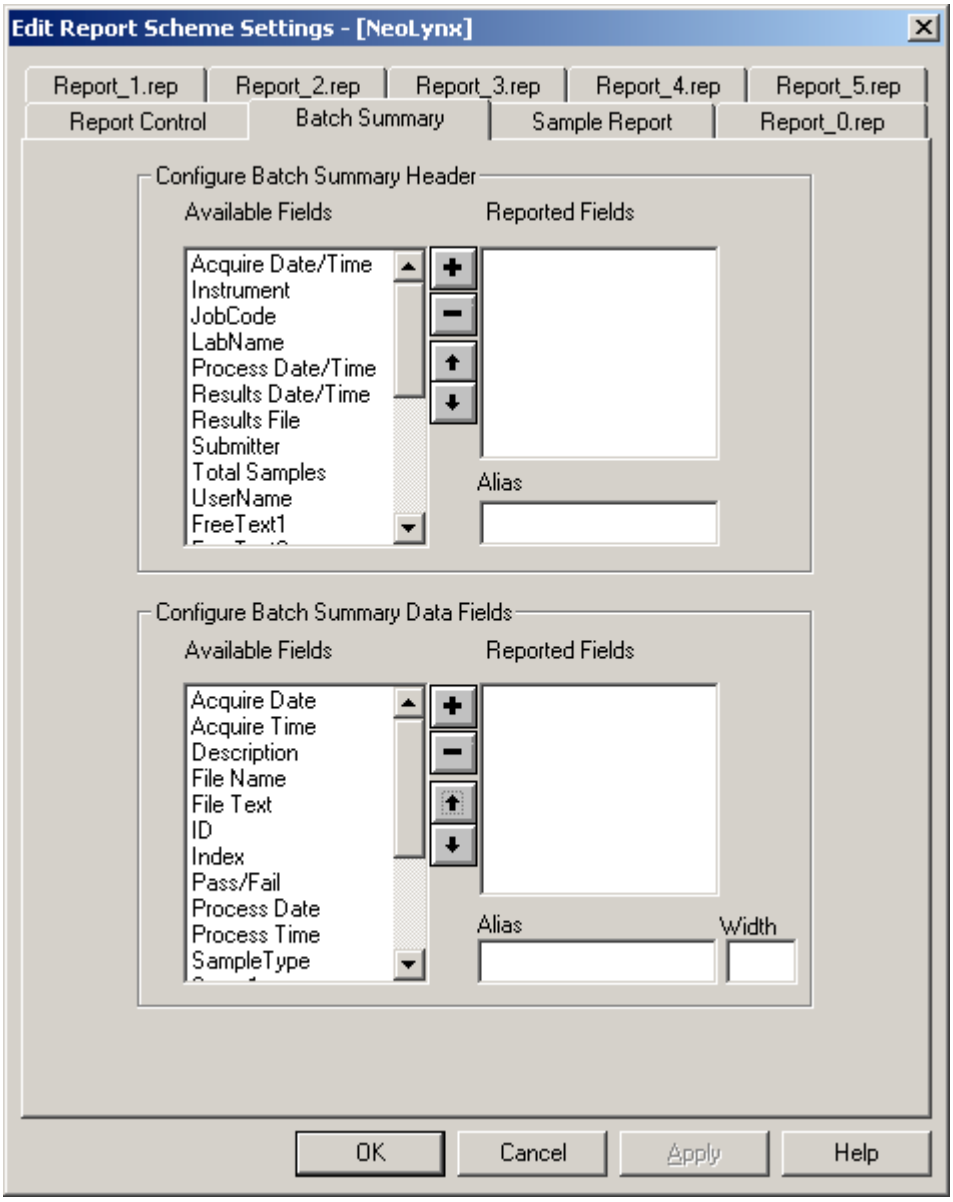


Figure 4.18 The Batch Summary Page

Batch Summary Header

This section defines the text printed in a Batch Summary Header. Twelve field areas are available in the Batch Summary Header. The available fields are all batch dependent, and may be selected from:

Acquire Date/Time The date and time of data acquisition.

Instrument The name or serial number of the instrument as defined in the MassLynx Control Panel.

Job Code The name of the Sample List from which the data were processed.

Lab Name The name of the Laboratory as defined in the MassLynx Control Panel.

Process Date/Time The date and time on which the data were processed.

Results Date/Time	The date and time at which the NRF file was created.
Results File	The path and name of the NRF file.
Submitter	The name of the person logged on to MassLynx at the time of data acquisition.
Total Samples	The total number of samples processed in the reported batch.
User Name	The name of the person logged on to MassLynx at the time of data processing.
Free Text 1 - 12	Twelve fields in which user-defined comments may be added.

Batch Summary Data Fields

The data reported in the columns of the Batch Summary are sample-dependent, the data fields available are:

Acquire Date	The date on which the file was acquired.
Acquire Time	The time at which the data file was acquired.
Description	The text string defined in the File Text column of the Sample List.
Filename	The name of the data file.
ID	The ID number defined in the ID column of the Sample List.
Index	The sequence number of the sample in the Sample List.
Pass/Fail	Whether the sample has Passed or Failed the tests in the NeoLynx Test File.
Process Date	The date on which the data file was processed.
Process Time	The time at which the data file was processed.
Sample Type	The Sample Type, as defined in the Sample List.
Spare 1-5	Information from the Spare columns in the Sample List.
Test File	The name of the NeoLynx Test File used to process the data.
Well	The Well location of the processed sample

To Add a Field

Click, on a field in the Available Fields box and press the  button. The new field is added to the bottom of the list.

To Remove a Field

Click, on the field to remove, in the Available Fields box and press the  button. The field will be removed from the list.

To Change the Order of Fields

Click on the field to move, in the Available Fields box and press the  or  button until the field is in the required position.

Field Alias

If the field name in the Available Fields list does not correspond to a description the user will recognize, another name can be used by entering it in the Field Alias box. E.g. Pass/Fail could be displayed on the report as State. This field may also be used to display field names in another language.

To Change the Field Width

Enter a new value in the Width box. **Note**: this option is not available for the Header fields.

NeoLynx Plate Report Generated by: Administrator All Samples Tested Page 1

Results File: PKU_MCA_001.nrf Total Samples: 34
Results File Created: 11-Sep-2000 09:49:01

Printed: Tue 10 Apr 2001 09:58:34

Plate Summary:

Symbols	
Standard Pass	0
Standard Fail	X
QC Pass	0
QC Fail	X
Analyte Pass	0
Analyte Fail	X
Not Used	-

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	X	X	X	X	X	X	X	0	0	X	X
B	X	0	0	X	X	-	-	-	-	-	-	-
C	0	X	X	0	X	X	X	X	0	0	0	X
D	X	0	0	X	X	-	-	-	-	-	-	-
F	-	-	-	-	-	-	-	-	-	-	-	-
G	-	-	-	-	-	-	-	-	-	-	-	-
H	-	-	-	-	-	-	-	-	-	-	-	-

Figure 4.19 3 A Plate Summary

NeoLynx Summary Report Generated by: Administrator All Samples Tested Page 1

Total Samples: 34 Acquire Date/Time: 19-May-2000 15:36:18 Instrument: QLC 9298
Results File: PKU_MCA_001.nrf Process Date/Time: 11-Sep-2000 09:49:01

Printed: Tue 10 Apr 2001 09:58:34

Sample Summary:

FileName	Pass/Fail	Well
PKU_MCA_001	Pass	1:A,1
PKU_MCA_002	**Fail**	**1:A,2**
PKU_MCA_003	**Fail**	**1:A,3**
PKU_MCA_004	**Fail**	**1:A,4**
PKU_MCA_005	**Fail**	**1:A,5**
PKU_MCA_006	**Fail**	**1:A,6**
PKU_MCA_007	**Fail**	**1:A,7**
PKU_MCA_008	**Fail**	**1:A,8**
PKU_MCA_009	Pass	1:A,9
PKU_MCA_010	Pass	1:A,10
PKU_MCA_011	**Fail**	**1:A,11**
PKU_MCA_012	**Fail**	**1:A,12**
PKU_MCA_013	**Fail**	**1:B,1**
PKU_MCA_014	Pass	1:B,2
PKU_MCA_015	Pass	1:B,3
PKU_MCA_016	**Fail**	**1:B,4**
PKU_MCA_017	**Fail**	**1:B,5**
PKU_MCA_018	Pass	1:C,1
PKU_MCA_019	**Fail**	**1:C,2**
PKU_MCA_020	Pass	1:C,3
PKU_MCA_021	**Fail**	**1:C,4**
PKU_MCA_022	**Fail**	**1:C,5**
PKU_MCA_023	**Fail**	**1:C,6**
PKU_MCA_024	**Fail**	**1:C,7**
PKU_MCA_025	**Fail**	**1:C,8**
PKU_MCA_026	Pass	1:C,9
PKU_MCA_027	Pass	1:C,10
PKU_MCA_028	Pass	1:C,11
PKU_MCA_029	**Fail**	**1:C,12**
PKU_MCA_030	**Fail**	**1:D,1**
PKU_MCA_031	Pass	1:D,2
PKU_MCA_032	Pass	1:D,3
PKU_MCA_033	**Fail**	**1:D,4**
PKU_MCA_034	**Fail**	**1:D,5**

Figure 4.20 A Batch Summary printout

The Sample Report Page

A Sample Report is a report relating to a single sample data file. Two distinct regions need to be configured - the **Sample Report Header**, and the **Sample Report Data Area**. The fields displayed on the report and the report header are selected in the same manner as for the Batch Summary (see above). It is also necessary to select the Sample Type (Standard, Analyte, QC) data to report.

An example of a printout is shown in Figure 4.22.

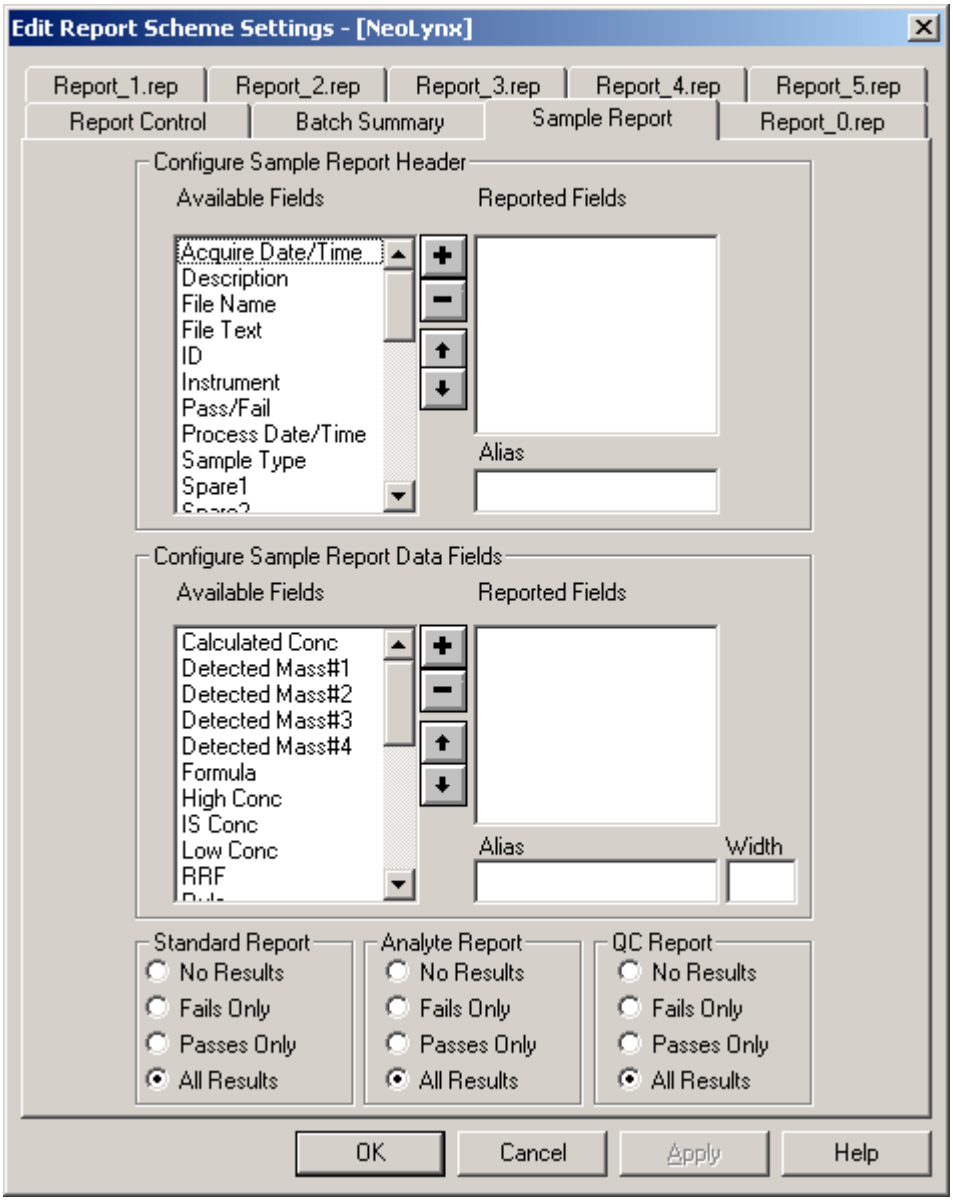


Figure 4.21 The Sample Report Page

The **Sample Summary Header** defines the text printed on a Sample Report Header and what information is printed on the Individual Sample Report. Twelve field areas are available in the Sample Summary Header.

The available fields are all sample-dependent, and may be selected from:-

Acquire Date/Time	The date and time on which the data were acquired.
Description	The text string defined in the File Text column of the Sample List.
Filename	The name of the data file.
ID	The ID number defined in the ID column of the Sample List.
Instrument	The name or serial number of the instrument as defined in the MassLynx Control Panel.
Pass/Fail	Whether the sample has Passed or Failed the tests in the NeoLynx Test File.
Process Date/Time	The date and time on which the data were processed.
Sample Type	The Sample Type, as defined in the Sample List.
Spare 1-5	Information from the Spare 1-5 columns in the Sample List.
Submitter	The name of the person logged on to MassLynx at the time of data acquisition.
Test File	The name of the NeoLynx Test File used to process the data.
User Name	The name of the person logged on to MassLynx at the time of data processing.
Well	The Well location of the processed sample
Free Text 1 - 11	Eleven fields in which user-defined comments may be added.

Sample Report Data Fields

The data reported in the columns of the Sample Summary are test-dependent, the data fields available are:

Calculated Conc	The Calculated Concentration from the Rule defined in the Test. Calculated Concentration = Ion Ratio * Multiplier.
Detected Mass 1-4	The masses detected and used for the calculations, according to Target Mass 1-4 and the defined Peak Detection Parameters.
Formula	The complete formula (Ion Ratio * Multiplier) defined in the Rule used in the Test.
High Conc	The value defined as the High Concentration in the Test.
IS Conc	The name of the Sample List from which the data were processed.
Low Conc	The value defined as the Low Concentration in the Test
RRF	The Relative Response Factor defined in the Test.
Rule	The name of the Rule used in the Test.

Spare 1-5 Information from the Spare columns in the Sample List.

Target Mass 1-4 The Target Mass(es) sought by the Test. These will be 0.00 if not required for the rule.

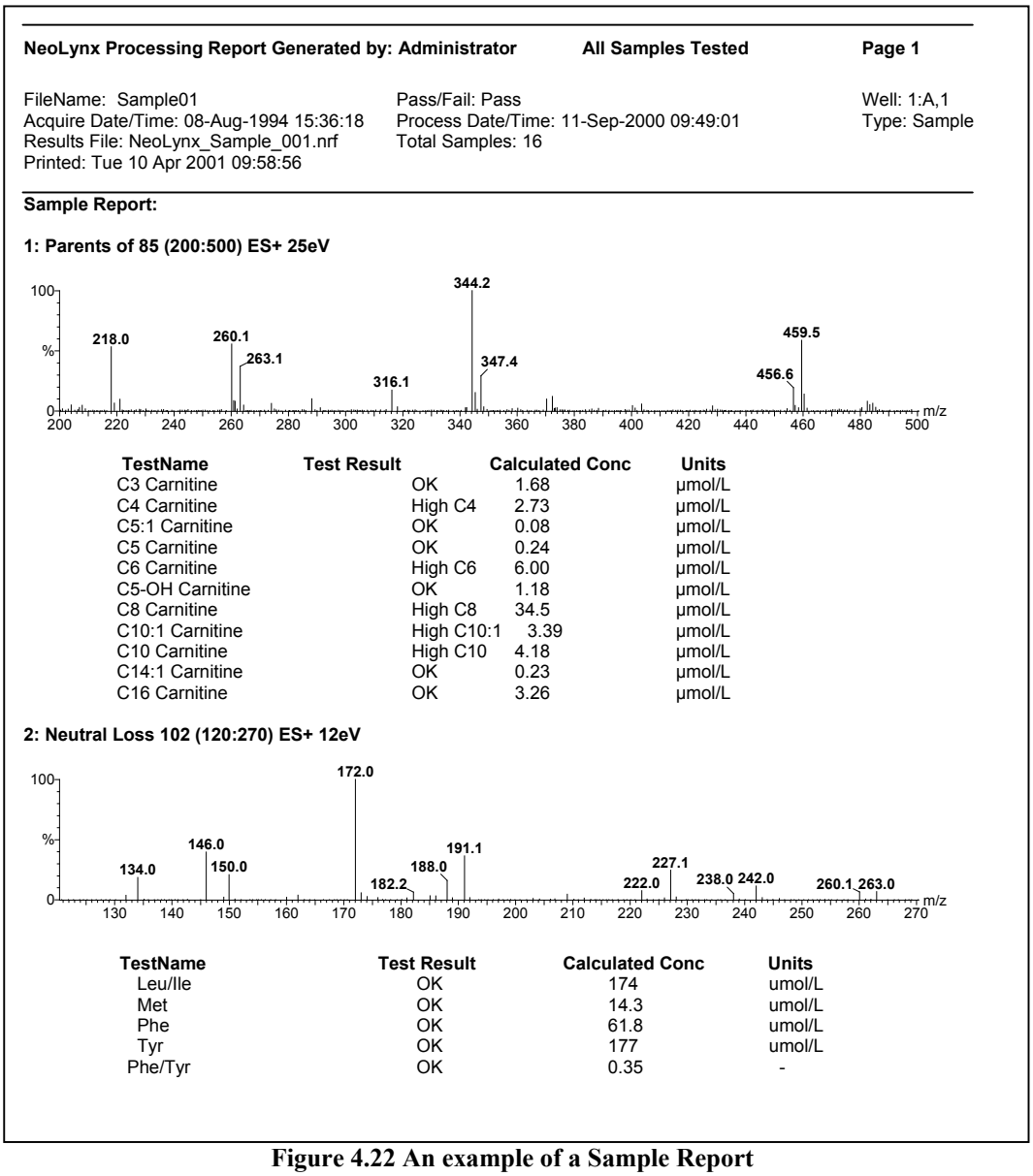
Test Name The name of the Test as defined in the NeoLynx Test File.

Test Result The result of the test - from Message Low, Message High or Message OK fields.

Units The units as defined in the designated Rule.

X The value of the X variable defined in the Test.

The lower region of the window shows three areas relating to different Sample Types. It is possible to differentiate between Sample Types, and only generate a Sample Report if the specified criteria are met. Available options are **No Results**, **Fails Only**, **Passes Only** or **All Results.**



NeoLynx Processing Report Generated by: Administrator **All Samples Tested** **Page 1**

FileName: Sample01 | Pass/Fail: Pass | Well: 1:A,1
Acquire Date/Time: 08-Aug-1994 15:36:18 | Process Date/Time: 11-Sep-2000 09:49:01 | Type: Sample
Results File: NeoLynx_Sample_001.nrf | Total Samples: 16
Printed: Tue 10 Apr 2001 09:58:56

Sample Report:

1: Parents of 85 (200:500) ES+ 25eV

TestName	Test Result	Calculated Conc	Units
C3 Carnitine	OK	1.68	µmol/L
C4 Carnitine	High C4	2.73	µmol/L
C5:1 Carnitine	OK	0.08	µmol/L
C5 Carnitine	OK	0.24	µmol/L
C6 Carnitine	High C6	6.00	µmol/L
C5-OH Carnitine	OK	1.18	µmol/L
C8 Carnitine	High C8	34.5	µmol/L
C10:1 Carnitine	High C10:1	3.39	µmol/L
C10 Carnitine	High C10	4.18	µmol/L
C14:1 Carnitine	OK	0.23	µmol/L
C16 Carnitine	OK	3.26	µmol/L

2: Neutral Loss 102 (120:270) ES+ 12eV

TestName	Test Result	Calculated Conc	Units
Leu/Ile	OK	174	umol/L
Met	OK	14.3	umol/L
Phe	OK	61.8	umol/L
Tyr	OK	177	umol/L
Phe/Tyr	OK	0.35	-

Figure 4.22 An example of a Sample Report

The Electronic Report Pages

In the NeoLynx Report Scheme supplied with NeoLynx 3.4 (NeoLynx.nrs), the electronic reports are configured to emulate the electronic reports supplied by default with NeoLynx 3.3. A summary of a report page is given here. Six different Electronic Report configurations are available. The default configurations, and the types of files generated are shown in Appendix E.

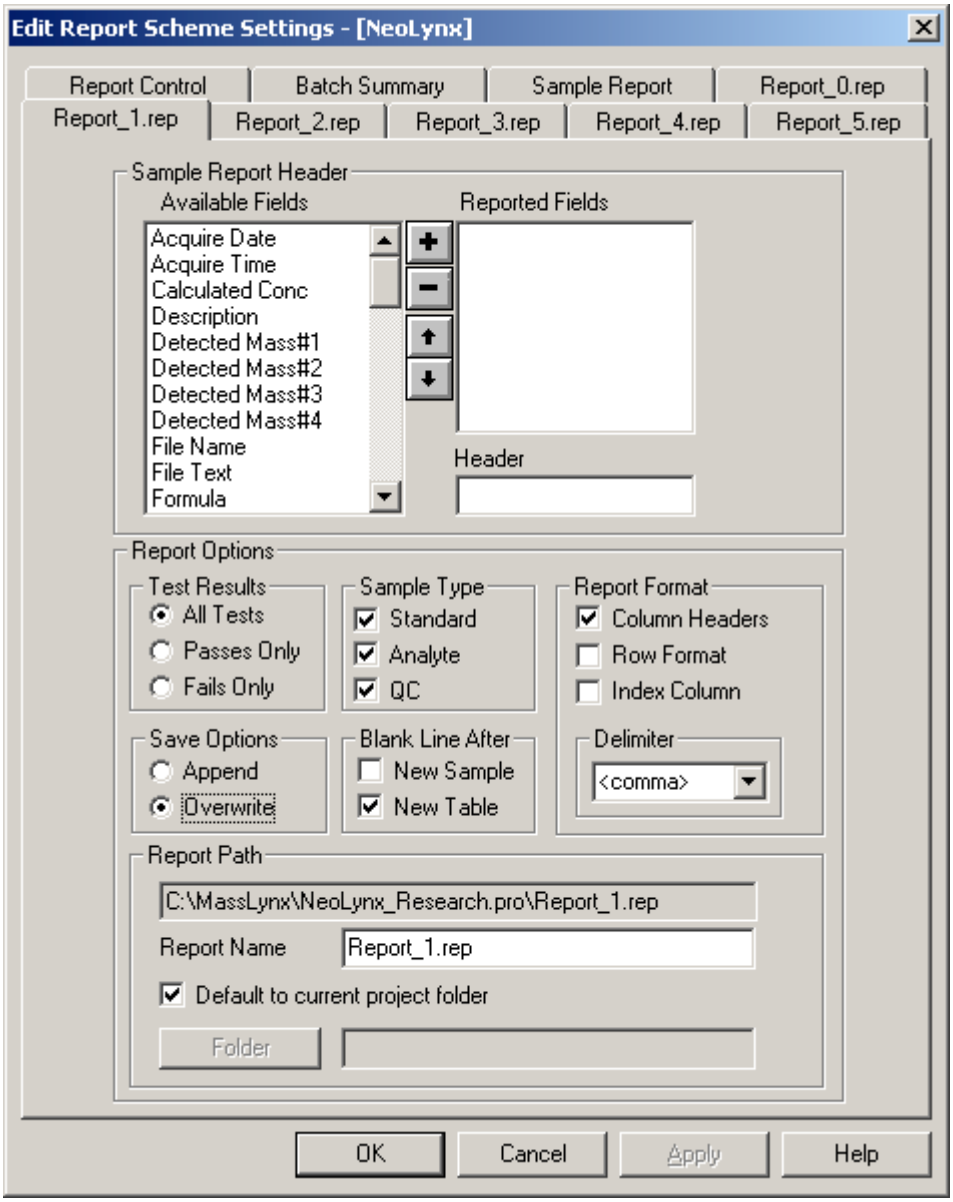


Figure 4.23 The Sample_Conc1 Page

The fields displayed on the report and the report header are selected in the same manner as for the Batch Summary. See The Batch Summary Page on page 4-24 for details.

The drop-down **Header** field allows for pre-defined column headers to be allocated if the **Column Headers** box is checked. User-defined column headers are also allowed.

Test Results Check one of All Tests, Passes Only or Fails Only to display the type of test result required.

Sample Type Check Standard, Analyte and/or QC to display the results for the required sample type(s).

Report Format Check Column Headers to show the column header on the first line of the report. Check the Row Format to show the results for a sample on one row. If Row Format is not selected each individual test result is printed in column format. Check Index Column to display the index field.

Save Options Check Append to add data to the end of the current report or Overwrite to create a new file. A report will be created if it does not already exist.

Blank Line After Check New Sample to print a blank line after each sample. Check New Table to print a blank line after a change of NeoLynx Test File in the Sample List..

Delimiter Select the delimiter, to use to separate fields, from the drop down list box. Available types are **tab**, **comma**, **space**, **semi-colon**, **none**. If a different delimiter is required type the single character in the Delimiter box.

Report Path To save the report to the C:\MASSLYNX directory check the **Default to current project folder** box. To write the report to a different location uncheck this box, press the **Folder** button and select a folder from the browser displayed.

Note that by using this option carefully, identical reports with identical names may be written to separate locations on a single computer, or across a network.

Report Name The name of the saved report is defined in this field. To incorporate the name of the Sample List from which the data was derived, enter %Sample% in the Report Name field as shown in Figure 4.23. The file extension may be user-defined. *.rep is used to reproduce the NeoLynx 3.3 Reports. It is possible to use other extensions e.g. *.xls, *.txt.

Selected References

This is a list of selected references that may be relevant in the use of mass spectrometry for neonatal screening, with particular focus on the amino acid and acylcarnitine metabolites. This list is not exhaustive for the period covered and is intended as a guide only.

1. Recent Developments in Neonatal Screening - E W Naylor, *Seminars in Perinatology*, 9 (1985) 232-249.

2. Newborn Screening: Past, Present and Future - R Guthrie, in *Genetic Disease: Screening and Management*, T P Carter and A M Wiley, Eds, Liss (1986) 319-339.

3. Hepatic Bile Acid Metabolism during Early Development Revealed from the Analysis of Human Fetal Gallbladder Bile - K D R Setchell, R Dumaswala, C Colombo and M Ronchi, *J. Biol. Chem.*, 263 (1988) 16637-16644.

4. Δ^4-3-Oxosteroid 5β-Reductase Deficiency Described in Identical Twins with Neonatal Hepatitis - K D R Setchell, F J Suchy, M B Welsh, L Zimmer-Nechemias, J Heubi and W F Balistreri, *J. Clin. Invest.*, 82 (1988) 2148-2157.

5. New-born Screening Fact Sheets - Committee on Genetics of the American Academy of Pediatrics, *Pediatrics*, 83 (1989) 449-464.

6. Identification of 3α,4β,7α trihydroxy-5β-cholanoic acid in Human Bile: Reflection of a New Pathway in Bile Acid Metabolism in Humans - R Dumaswala, K D R Setchell, L Zimmer-Nechemias, T Iida, J Goto and T Nambara, *J. Lipid Res.*, 30 (1989) 847-856.

7. Acyl-CoA Dehydrogenase Deficiencies - C R Roe and P M Coates, *Metabolic Basis of Inherited Diseases*, McGraw Hill (1989) 889-914.

8. Dienoyl-Coenzyme A Reductase Deficiency : A Possible New Disorder of Fatty Acid Oxidation - C R Roe, D S Millington, D L Norwood, N Kodo, H Sprecher, B S Mohammed, M Nada, H Schulz and R McVie, *J. Clin. Invest.*, 85 (1990) 1703-1707.

9. Comprehensive Study of the Biliary Bile Acid Composition of Patients with Cystic Fibrosis and Associated Liver Disease Before and After UDCA Administration - M Nakagawa, C Colombo and K D R Setchell, *Hepatology*, 12 (1990) 322-334.

10. Tandem Mass Spectrometry: A New Method for Acylcarnitine Profiling with Potential for Neonatal Screening for Inborn Errors of Metabolism - D S Millington, N Kodo, D L Norwood and C R Roe, *J. Inher. Metab. Dis.*, 13 (1990) 321-324.

11. Bile Acid Metabolism in Early Life: Studies of Amniotic Fluid - M Nakagawa and K D R Setchell, *J. Lipid Res.*, 31 (1990) 1089-1098.

12. The Analysis of Diagnostic Markers of genetic Disorders in Human Blood and Urine using Tandem Mass Spectrometry with Liquid Secondary Ion Mass Spectrometry - D S Millington, N Kodo, N Terada, D Roe and D H Chace, *Int. J. Mass Spectrom. Ion Processes*, 111 (1991) 211-228.

13. New Technologies in New-born Screening - E W Naylor, *Yale J. Biol. Med.* 64 (1991) 21-24.

14. Failure of Ursodeoxycholic Acid to Prevent a Cholestatic Episode in a Patient with Benign Recurrent Intrahepatic Cholestasis: A Study in Bile Acid Metabolism - A Crosignani, M Podda, E Bertolini, P M Battezzati, M Zuin and K D R Setchell, *Hepatology*, 13 (1991) 1076-1083.

15. The Role of Tandem Mass Spectrometry in the Diagnosis of Fatty Acid Oxidation Disorders - D S Millington, N Terada, D H Chace, Y-T Chen, J-H Ding, N Kodo and C R Roe, *New Developments in Fatty Acid Oxidation*, Wiley-Liss (1992) 339-354.

16. Carnitine and Acylcarnitines in Metabolic Disease Diagnosis and Management - D S Millington and D H Chace, in *Mass Spectrometry: Clinical and Biomedical Applications Vol. 1*, D M Desiderio, Ed., Plenum (1992) 299-318.

17. Carnitine and Acylcarnitine Analysis in the Diagnosis of Metabolic Diseases: Advantages of Tandem Mass Spectrometry - D S Millington, N Terada, N Kodo and D H Chace, in *Advances in Chemical Diagnosis and Treatment of Metabolic Disorders, Volume 1*, I Matsumoto, Ed., Wiley (1992) 59-71.

18. Neonatal Screening for Duchenne/Becker Muscular Dystrophy; Reconsideration Based on Molecular Diagnosis and Potential Therapeutics - E W Naylor, E P Hoffman, J Paulus-Thomas, H B Wessel, K S Reid, B Mitchell and B J Schmidt, *Screening*, 1 (1992) 99-113.

19. Oral Bile Acid Treatment and the Patient with Zellweger Syndrome - K D R Setchell, P Bragetti, L Zimmer-Nechemias, C Daugherty, M A Pelli, R Vaccaro, G Gentili, E Distrutti, G Dozzini, A Morelli and C Clerici, *Hepatology*, 15 (1992) 198-207.

20. Rapid Diagnosis of Phenylketonuria by Quantitative Analysis for Phenylalanine and Tyrosine in Neonatal Blood Spots by Tandem Mass Spectrometry - D H Chace, D S Millington, N Terada, S G Kahler, C R Roe and L F Hofman, *Clin. Chem.*, 39 (1993) 66-71.

21. Medium-Chain Acyl-CoA Dehydrogenase (MCAD) Deficiency: Diagnosis by Acylcarnitine Analysis in Blood - J L K van Hove W Zhang, S G Kahler, C R Roe, Y-T Chen, N Terada, D H Chace, A K Iafolla, J-H Ding and D S Millington, *Am. J. Hum. Genet.*, 52 (1993) 958-966.

22. Neonatal Screening for Cystic Fibrosis: Addition of Molecular Diagnostics to Increase Specificity - W C Spence, J Paulus-Thomas, D M Orenstein and E W Naylor, *Biochem. Med. Metab. Biol.*, 49 (1993) 200-211.

23. The Analysis of Acylcarnitines - B M Kelly, M E Rose and D S Millington, in *Advances in Methodology - Two*, W W Christie, Ed., The Oily Press, (1993) 247-289.

24. Ion spray liquid chromatography / mass spectrometric characterization of bile acids - B M Warrack and G C DiDonato, *Biol. Mass Spectrom.*, 22 (1993) 101-111.

25. Screening techniques for the detection of inborn errors of bile acid metabolism by direct injection and micro-high performance liquid chromatography-continuous flow / fast atom bombardment mass spectrometry - J E Evans, A Ghosh, B A Evans and M R Natowicz, *Biol. Mass Spectrom.*, 22 (1993) 331-337.

26. 3-Hydroxydicarboxylic and 3-Ketodicarboxylic Aciduria in Three Patients: Evidence for a New Defect in Fatty Acid Oxidation at the Level of 3-Ketoacyl-CoA Thiolase - M J Bennett and W G Sherwood, *Clin.Chem.*, 39 (1993) 897-901.

27. Dietary Supplement L-Carnitine: Analysis of Different Brands to Determine Bioavailability and Content - D S Millington and G Dubay, *Clin. Res. Reg. Affairs*, 10 (1993) 71-80.

28. Electrospray Tandem Mass Spectrometry in the Analysis of Organic Acidemias - M S Rashed, P T Ozand, M E Harrison, P J F Watkins and S Evans, *Rapid Commun. Mass Spectrom.*, 8 (1994) 129-133.

29. Neonatal Screening for Inborn Errors of Metabolism by Automated Dynamic Liquid Secondary Ion Tandem Mass Spectrometry - D H Chace and D S Millington, in *New Horizons in Neonatal Screening*, J-P Farriaux and J-L Dhondt, Eds, Elsevier (1994) 373-376.

30. Inborn Errors of Bile Acid Metabolism - K D R Setchell and N C O'Connell in *Liver Disease in Children*, F J Suchy, Ed, (1994) 835-851.

31. Diagnosis of Metabolic Disease - D S Millington, D H Chace, S L Hillman, N Kodo and N Terada, in *Biological Mass Spectrometry: Present and Future*, T Matsuo, Y Seyama, R M Caprioli and M L Gross, Eds, Wiley (1994) 559-579.

32. Δ^4-3-Oxosteroid 5β-reductase deficiency causing neonatal liver failure and hemochromatosis - B L Shneider, K D R Setchell, P F Whitington, K A Nellson and F J Suchy, *J. Pediatr.*, 124 (1994) 234-238.

33. A new cause of progressive intrahepatic cholestasis: 3β-hydroxy-C_{27}-steroid dehydrogenase / isomerase deficiency - E Jacquemin, K D R Setchell, N C O'Connell, A Estrada, G Maggiore, J Schmitz, M Hadchouel and O Bernard, *J. Pediatr.*, 125 (1994) 379-384.

34. Tiglylglycine Excreted in Urine in Disorders of Isoleucine Metabolism and the Respiratory Chain Measured by Stable Isotope Dilution GC-MS - M J Bennett, S Powell, D J Swartling and K M Gibson, *Clin. Chem.*, 40 (1994) 1879-1883.

35. Inherited Defects of Fatty Acid Oxidation - C R Roe and M A Nada, in *Fatty Acids and Lipids: Biological Aspects*, G C Simopolous and E Tremoli, Eds, Karger (1994) 22-25.

36. Automated ESI-MS/MS using 96-Well Microplate Batch Processing for Rapid High-Throughput Metabolic Profiling of the Butyl Derivatives of Free Carnitine, Acylcarnitines and Amino Acids. - M Rashed, M Bucknall, A Awad, M Jacob, D Little, M Al-Amoudi and P Ozand, Presented at the 3rd International Symposium on Applied Mass Spectrometry in the Health Sciences, Barcelona, June 1995.

37. Diagnosis of Inborn Errors of Metabolism from Blood Spots by Acylcarnitines and Amino Acids Profiling using Automated Electrospray Tandem Mass Spectrometry - M S Rashed, P T Ozand, M P Bucknall and D Little, *Pediatr. Res.*, 38 (1995) 324-331.

38. Glycine and L-Carnitine Therapy in 3-Methyl-crotonyl-CoA carboxylase Deficiency - S L Rutledge, G T Berry, C A Stanley, J L K van Hove and D Millington, *J. Inher. Metab. Dis.*, 18 (1995) 299-305.

39. Rapid Diagnosis of Maple Syrup Urine Disease in Blood Spots from Newborns by Tandem Mass Spectrometry - D H Chace, S L Hillman, D S Millington, S G Kahler, C R Roe and E W Naylor, *Clin. Chem.*, 41 (1995) 62-68.

40. The Role of Tandem Mass Spectrometry in Reducing the Number of False Positive and False Negative Results in the Diagnosis of Metabolic Disease from Dried Blood Spots - D H Chace, D S Millington and S L Hillman, in *Early Hospital Discharge: Impact on Newborn Screening*, K A Pass and H L Levy, Eds, (1995) 272-283.

41. High-performance Liquid Chromatographic - Electrospray Mass Spectrometric Analysis of Bile Acids in Biological Fluids - A Roda, A N Gioacchini, C Cerrè and M Baraldini, *J. Chromatogr. B*, 665 (1995) 281-294.

42. Inborn Errors of Metabolism Diagnosed in Sudden Death Cases by Acylcarnitine Analysis of Postmortem Bile - M S Rashed, P T Ozand, M J Bennett, J J Barnard, D R Govindaraju and P Rinaldo, *Clin Chem.*, 41 (1995) 1109-1114.

43. Evidence for Intermediate Channeling in Mitochondrial β-Oxidation - M A Nada, W J Rhead, H Sprecher, H Schulz and C R Roe, *J. Biol. Chem.*, 270 (1995) 530-535.

44. Δ^{22}-ursodeoxycholic Acid, a Unique Metabolite of Administered Ursodeoxycholic Acid in Rats, Indicating Partial β-Oxidation as a Major Pathway for Bile Acid Metabolism - K D R

Setchell, H Yamashita, C M P Rodrigues, N C O'Connell, B T Kren and C J Steer, *Biochemistry*, 34 (1995) 4169-4178.

45. Investigation of β-Oxidation Intermediates in Normal and MCAD-Deficient Human Fibroblasts using Tandem Mass Spectrometry - M A Nada, D H Chace, H Sprecher and C R Roe, *Biochem. Molec. Medicine*, 54 (1995) 59-66.

46. Medium Chain Acyl-CoA Dehydrogenase Deficiency in Pennsylvania: Neonatal Screening Shows High Incidence and Unexpected Mutation Frequencies - R Ziadeh, E P Hoffman, D N Finegold, R C Hoop, J C Brackett, A W Strauss and E W Naylor, *Pediatr. Res.*, 37 (1995) 675-678.

47. Rapid Diagnosis of Homocystinuria and other Hypermethioninemias from Newborns' Blood Spots by Tandem Mass Spectrometry - D H Chace, S L Hillman, D S Millington, S G Kahler, B W Adam and H L Levy, *Clin. Chem.*, 42 (1996) 349-355.

48. Disorders of Bile Acid Synthesis and Metabolism - K D R Setchell, in *Pediatric Gastrointestinal Disease, Pathophydiology, Diagnosis, Management*, W A Walker, P R Durie, J R Hamilton, J A Walker-Smith and J A Watkns, Eds, (1996) 1205-1233.

49. Newborn Screening by Tandem Mass Spectrometry (MS-MS) - L Sweetman, *Clin. Chem.*, 42 (1996) 345-346.

50. Rapid Diagnosis of MCAD Deficiency: Quantitative Analysis of Octanoylcarnitine and Other Acylcarnitines in Newborn Blood Spots by Tandem Mass Spectrometry - D H Chace, S L Hillman, J L K van Hove and E W Naylor, *Clin. Chem.*, 43 (1997) 2106-2113.

51. Additive Hypocholesterolemic Effect of Psyllium and Cholestyramine in the Hamster: Influence in Fecal Sterol and Bile Acid Profiles - B P Daggy, N C O'Connell, G R Jerdack, B A Stinson and K D R Setchell, *J. Lipid res.*, 38 (1997) 491-502.

52. Screening Blood Spots for Inborn Errors of Metabolism by Electrospray Tandem Mass Spectrometry with a Microtitre Plate Batch Process and a Computer Algorithm for Automated Flagging of Abnormal Profiles - M S Rashed, M P Bucknall, D Little, A Awad, M Jacob, M Alamoudi, M Alwattar and P T Ozand, *Clin. Chem.*, 43 (1997) 1129-1141.

53. A Method for the Quantitation of Conjugated Bile Acids in Dried Blood Spots using Electrospray Ionisation Mass Spectrometry - K A Mills, I Mushtaq, A W Johnson, P D Whitfield and P T Clayton, *Pediatr. Res.*, 43 (1998) 361-368.

54. Recoveries of Phenylalanine from two sets of dried blood spots reference materials: Prediction from Hematocrit, Spot Volume and Paper Matrix - B W Adam, J R Alexander, S J Smith, D H Chace, J G Loeber, L H Elvers and W H Hannon, *Clin. Chem.*, 46 (2000) 126-127.

55. Effect of Storage on Phenylalanine and Tyrosine Measurements in Whole-Blood Samples, M Beck, A Bökenkamp, N Liappis and M J Lentze, *Clin. Chem.*, 47 (2001) 751-752.

56. The Metabolic Autopsy Comes of Age, M J Bennett, *Clin. Chem.*, 47 (2001) 1145-1146.

57. Rapid Determination Total Homocysteine in Blood Spots by Liquid Chromatography - Electrospray Ionisation - Tandem Mass Spectrometry, K Gempel, K-D Gerbitz, B Casetta and M F Bauer, *Clin. Chem.*, 46(2000) 122-123.

58. The use of phenylalanine-to-tyrosine ratio determined by tandem mass spectrometry to improve newborn screening for pheylketonuria or early discharge specimens collected in the first 24 hours, D H Chace, J E Sherwin, S L Hillman, F Lorey and G C Cunningham, *Clin. Chem.*, 44 (1998) 2405-2409.

59. Validation of Accuracy-based Amino Acid Reference Materials in Dried-Blood Spots by Tandem Mass Spectrometry for Newborn Screeing Analysis, D H Chace, B W Adam, S J Smith, J R Alexander, S L Hillman and W H Hannon, *Clin. Chem.*, 45 (1999) 1269-1277.

60. Electrospray Tandem Mass Spectrometry for Analysis of Acylcarnitines in Dried Postmortem Blood Specimens Collected at Autopsy from Infants with Unexplained Cause of Death, D H Chace, J C DiPerna, B L Mitchell, B Sgroi, L F Hofman and E W Naylor, *Clin. Chem.*, 47 (2001) 1166-1182.

61. Errors caused by the use of D,L-Octanoylcarnitine for Blood-Spot Calibrators, D H Chace, J C DiPerna, B W Adam and W H Hannon, *Clin. Chem.*, 47 (2001) 758-760.

62. Neonatal Screening for Galactosemia by Quantitative Analysis of Hexose Monophosphates using Tandem Mass Spectrometry: A Retrospective Study, U G Jensen, N J Brandt, E Christensen, F Skovby, B Nørgaard-Pedersen and H Simonsen, *Clin. Chem.*, 47 (2001) 1364-1372.

63. Method for the Determination of Total Homocysteine in Plasma and Urine by Stable Isotope Dilution and Electrospray Tandem Mass Spectrometry, M J Magera, J M Lacey, B Casetta and P Rinaldo, *Clin. Chem.*, 45 (1999) 1517-1522.

64. Methylmalonic Acid Measured in Plasma and Urine by Stable-Isotope Dilution and Electrospray Tandem Mass Spectrometry, M J Magera, J K Helgeson, D Matern and P Rinaldo, *Clin. Chem.*, 46 (2000) 1804-1810.

65. Assay from Free and Total Carnitine in Human Plasma using Tandem Mass Spectrometry, R D Stevens, S L Hillman, S Worthy, D Sanders and D S Millington, *Clin. Chem.*, 46 (2000) 727-729.

66. Newborn Screening by Tandem Mass Spectrometry, L Sweetman, *Clin. Chem.*, 47 (2001) 1937-1938.

67. Phenylalanine and Tyrosine Quantification by Stable Isotope Dilution Liquid Chromatography - Mass Spectrometry from Filter Paper Blood Spots, M Tuchman and M T McCann, *Clin. Chem.*, 45 (1999) 571-573.

68. Defects in Pyrimidine Degradation Identified by HPLC-Electrospray Tandem Mass Spectrometry of Urine Specimens or Urine-soaked Filter Paper Strips, H vanLenthe, A B P van Kuilenburg, T Ito, A H Bootsma, A van Crutchen, Y Wada and A H van Gennip, *Clin. Chem.*, 46 (2000) 1916-1922.

69. Rapid Screening of High-Risk Patients for Disorders of Purine and Pyrimidine Metabolism Using HPLC-Electrospray Tandem Mass Spectrometry of Urine Specimens or Urine-soaked Filter Paper Strips, T Ito, A B P van Kuilenburg, A H Bootsma, A J Haasnoot, A van Crutchen, Y Wada and A H van Gennip, *Clin. Chem.*, 46 (2000) 1916-1922.

70. Tandem Mass Spectrometric Analysis for Amino, Organic and Fatty Acid Disorders in Newborn Dried Blood Spots: A Two-Year Summary from the New England Newborn Screening Program, T H Zytkovicz, E F Fitzgerald, D Marsden, C A Larson, V E Shih, D M Johnson, A W Strauss, A M Comeau, R B Eaton and G F Grady, *Clin. Chem.*, 47 (2001) 1945-1955.

71. Evaluation of Newborn Screening for Medium-Chain Acyl-CoA Dehydrogenase Deficiency in 275,000 Babies, K Carpenter, V Wiley, K G Sim, D Heath and B Wilcken, *Arch. Dis. Child. Fetal Neonatal*, 85 (2001) F105-F109.

72. Quantitative Plasma Acylcarnitine Analysis using Electrospray Tandem Mass Spectrometry for the Diagnosis of Organic Acidaemias and Fatty Acid Oxidation Defects, P Vreken, A E M avn Lint, A H Bootsma, H Overmars, R J A Wanders and A H van Gennip, *J. Inher. Metab. Dis.*, 22 (1999) 302-306.

73. Screening of Newborn Infants for Cholestatic Hepatobiliary Disease with Tandem Mass Spectrometry, I Mushtaq, S Logan, M Morris, A W Johnson, A M Wade, D Kelly and P T Clayton, *Br. Med. J.*, 319 (1999) 471-477.

74. Rapid Analysis of Conjugate Bile Acids in Plasma using Electrospray Tandem Mass Spectometry: Application for Selective Screening of Peroxisomal Disorders, *J. Inher. Metab. Dis.*, 22 (1999) 307-310.

75. Clinical Applications of Tandem Mass Spectrometry: Ten Years of Diagnosis and Screening for Inherited Metabolic Disease, M S Rashed, *J Chro. B*, 758 (2001) 27-48.

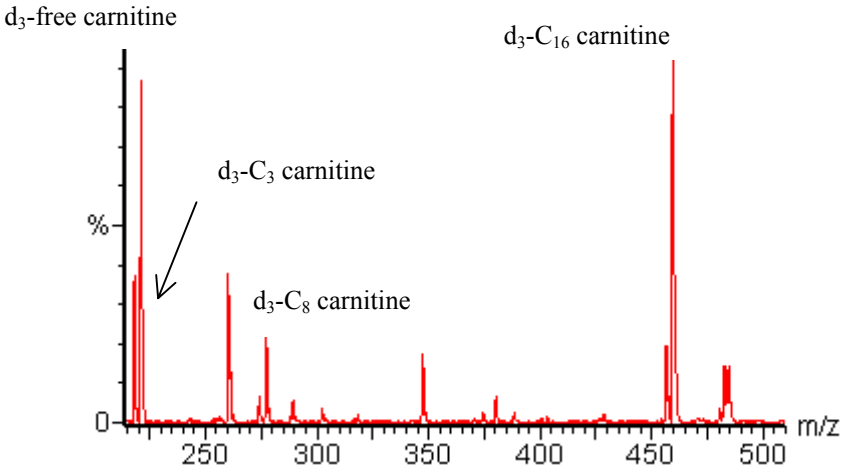
Appendix A Data Compendium

The data shown in the following pages are for illustrative purposes only. The intention of these inclusions is to give the reader examples of the types of data that may be seen during the analysis spots. This compendium is by no means comprehensive.

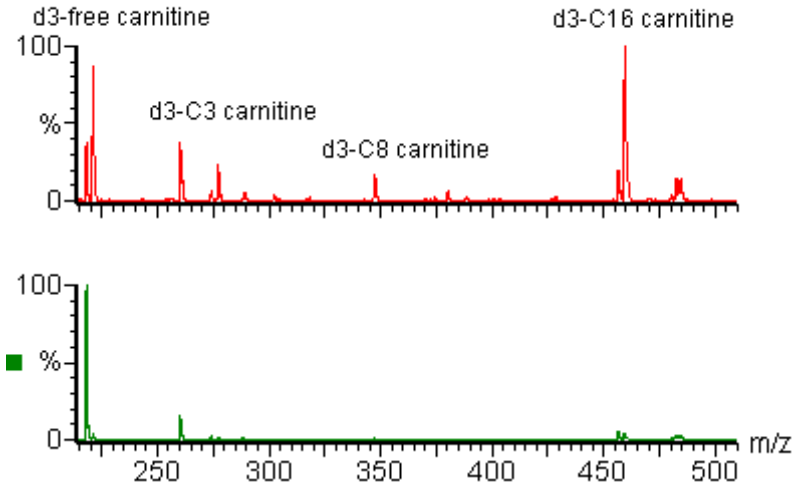
The data were acquired as part of a demonstration for Dr Mohamed Rashed of the King Faisal Specialist Hospital and Research Center, Riyadh, and were acquired on a Quattro LC mass spectrometer coupled to an HP1100/Gilson 215 combination.

Selected acylcarnitine profiles

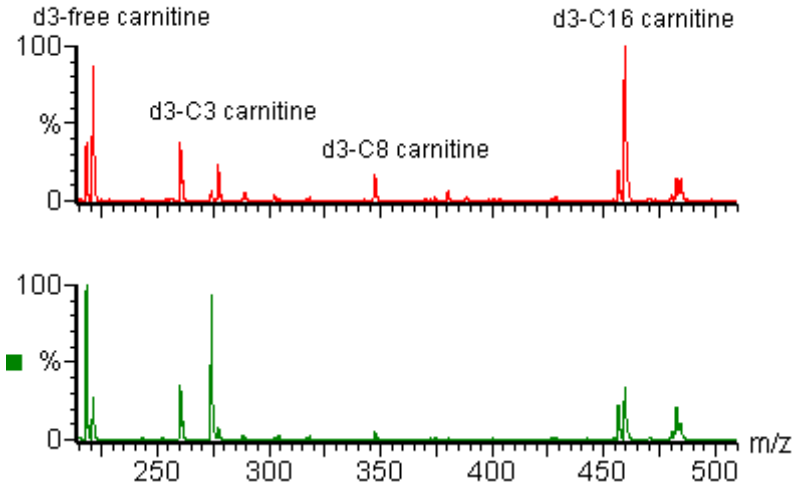
Function type:	Parents of m/z 84.80
Mass range:	m/z 215 to m/z 510
Cone Voltage:	30 V
Collision Energy:	25.0 eV
Collision gas pressure:	1.4 x 10^{-3} mbar Argon
Scan time:	3.0 secs (0.05 sec inter-scan delay)



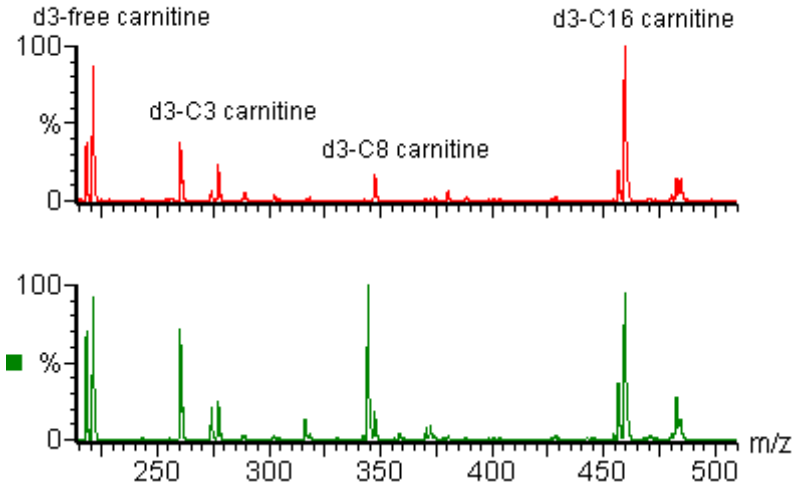
Typical acylcarnitine profile from a healthy patient.



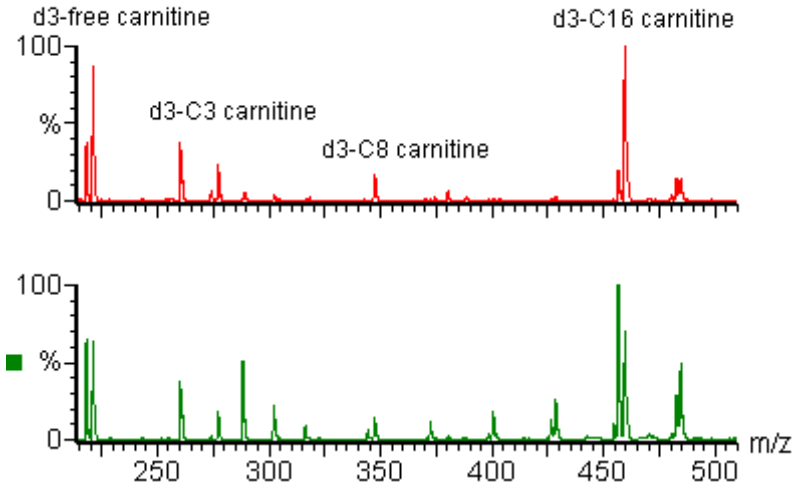
Normal trace *vs* patient with significant elevation of free carnitine



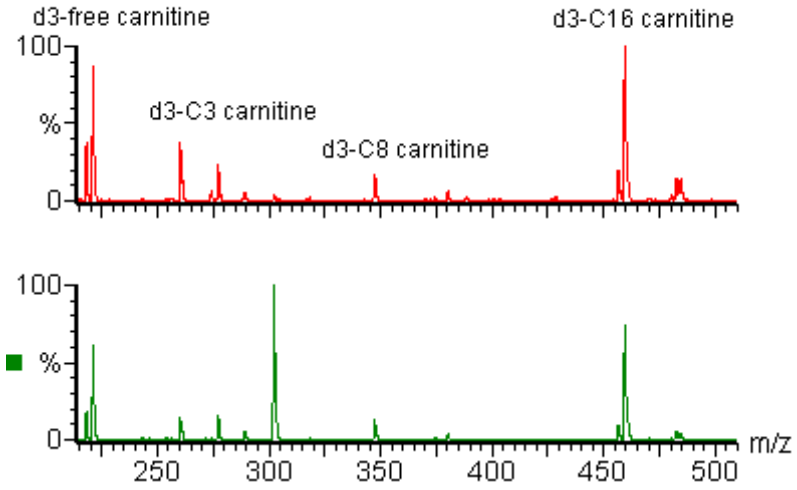
Normal patient *vs* patient with significant elevation of C3-carnitine. Indicative of propionic acidaemia or methyl malonic acidaemia.



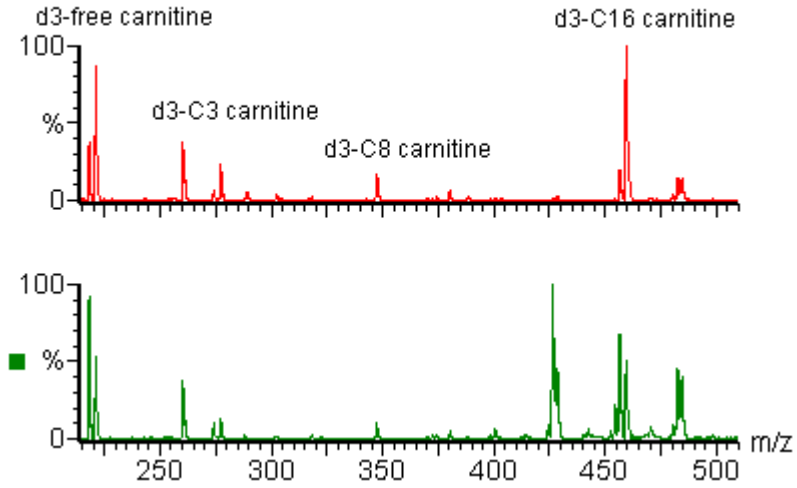
Normal patient *vs* patient with elevated medium chain (C6, C8, C10, C10:1) acylcarnitines. The patient from the lower trace was diagnosed with MCAD.



Normal patient *vs* patient showing elevations of a broad spectrum of acylcarnitines. Indicative of multiple acyl-CoA dehydrogenase deficiency or glutaric acidaemia type II.



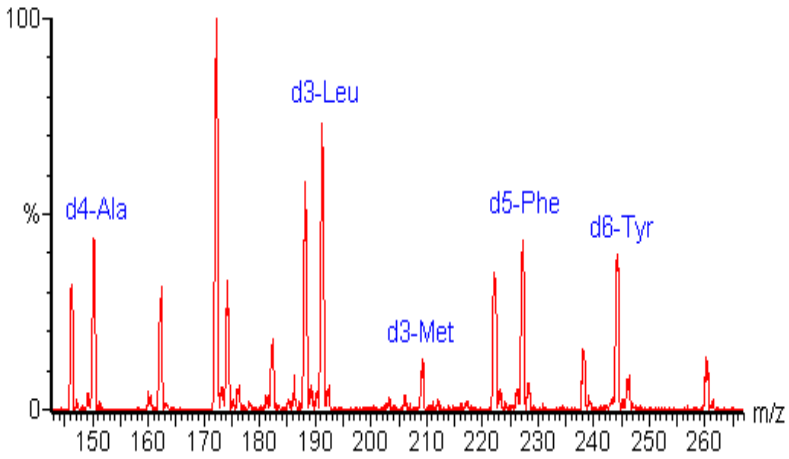
Normal patient *vs* patient with elevated isovaleryl carnitine.



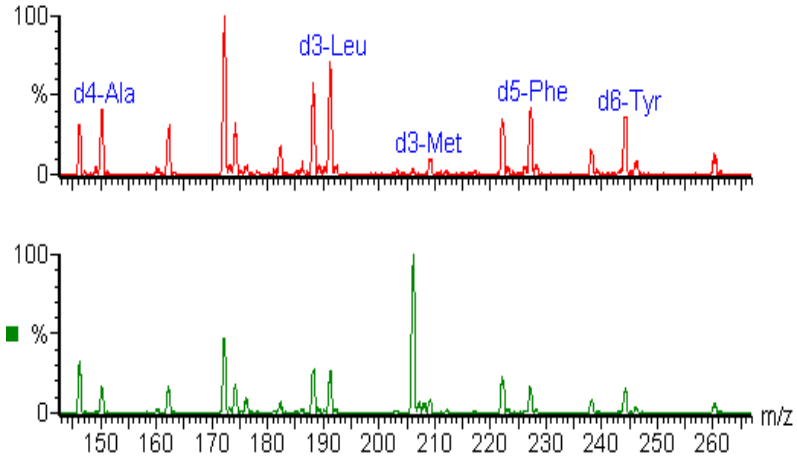
Normal patient *vs* patient showing elevation of the very long chain (C14-C18) acylcarnitines. Diagnosis of long-chain acyl-CoA dehydrogenase deficiency is not as clear cut from tandem mass spectra as it is with some other disorders. Further testing would be indicated.

Selected neutral / acidic amino acid profiles

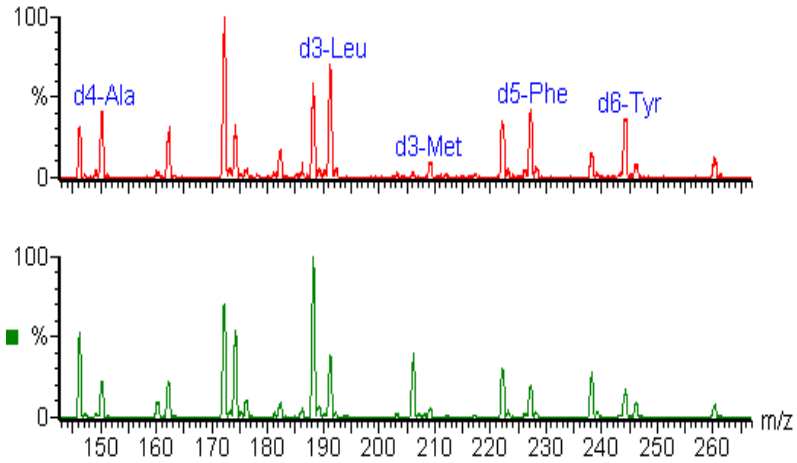
Function type:	Neutral Loss of 102.10 Da
Mass range:	m/z 125 to m/z 300
Cone Voltage:	25 V
Collision Energy:	10.0 eV
Collision gas pressure:	1.4 x 10^{-3} mbar Argon
Scan time:	0.9 secs (0.05 sec inter-scan delay)



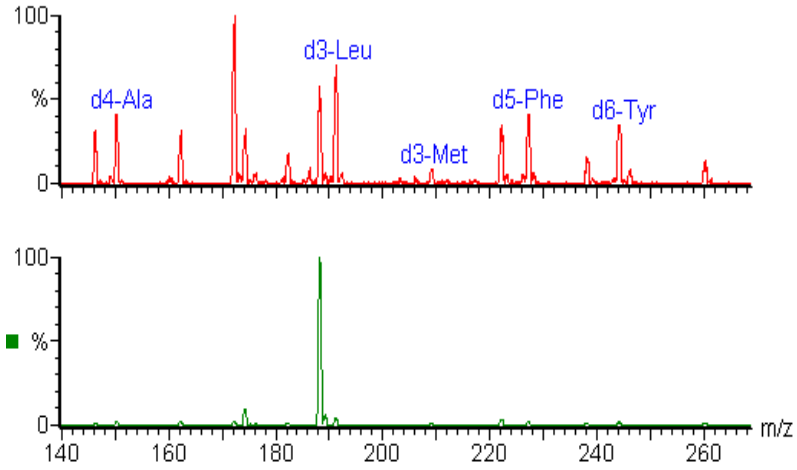
Typical neutral / acidic amino acid profile from a healthy patient.



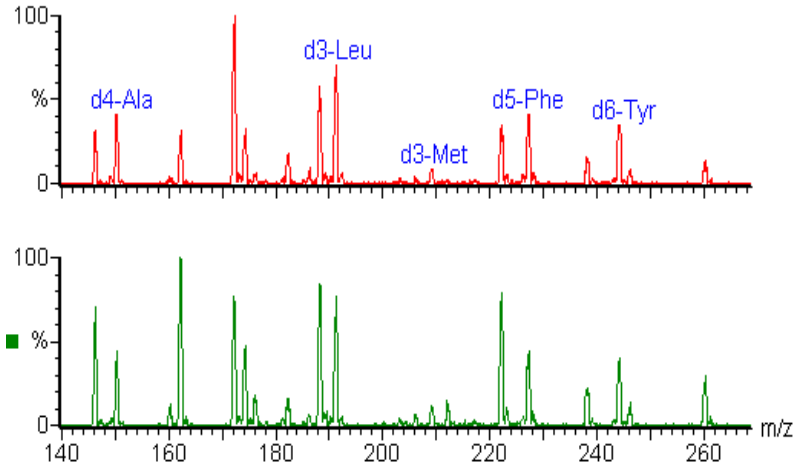
Normal patient *vs* patient with elevated methionine (*m/z* 206).



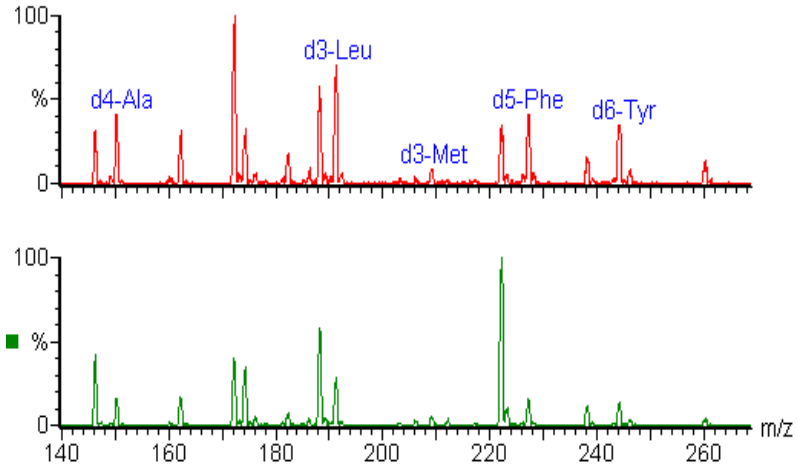
Normal patient *vs* patient with elevated Leu/Ile (*m/z* 188). Note that it is not possible to differentiate the leucine isomers in a simple tandem mass spectrometry experiment - additional chromatographic separation would be necessary for absolute determinations.



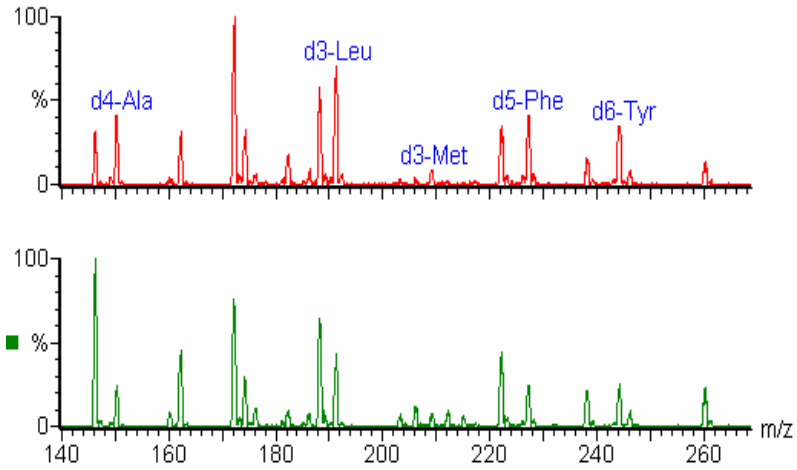
Normal patient *vs* patient with severely elevated Leu/Ile (*m/z* 188) from an uncontrolled maple syrup urine disease (MSUD). Note the difference between this spectrum and the one above showing the different levels of quantification that can be achieved in these experiments.



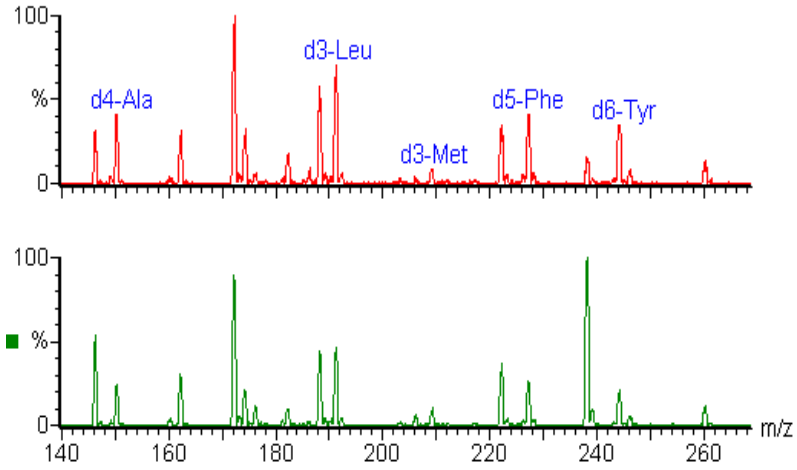
Normal patient *vs* patient with slightly elevated Phe (*m/z* 222).



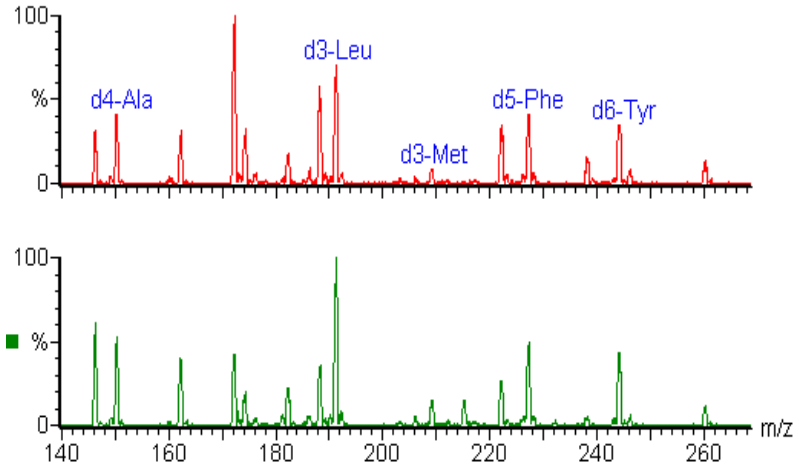
Normal patient *vs* patient with severely elevated Phe (*m/z* 222) in uncontrolled phenylketonuria (PKU).



Normal patient *vs* patient with elevated Ala (*m/z* 146).



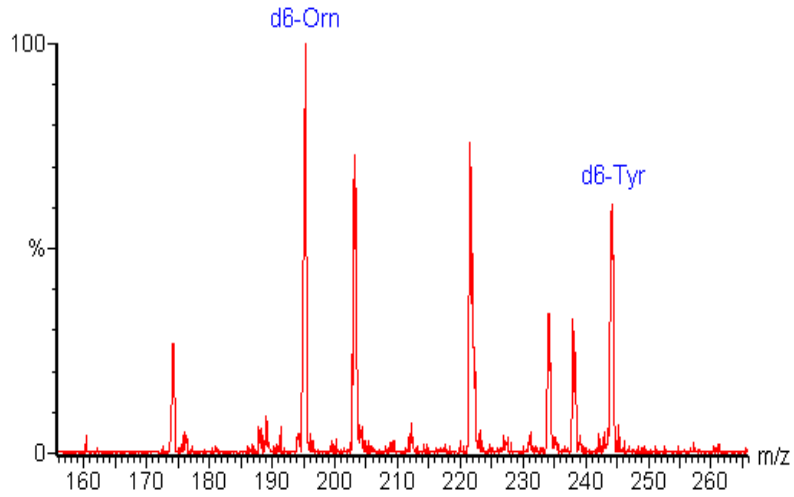
Normal patient *vs* patient with elevated levels of Tyr (*m/z* 238). Absolute determination of elevated levels of tyrosine is difficult as a number of cases have been reported in which transient neonatal tyrosinaemia has been observed.



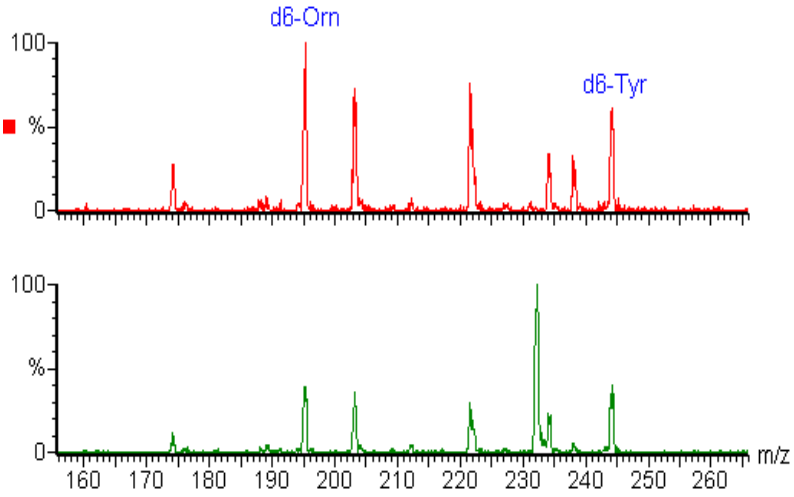
Normal patient *vs* patient with elevated citrulline (*m/z* 215). Although citrulline is a basic amino acid, it can often be detected in the neutral / acidic amino acid scan function under the scanning conditions used.

Selected basic amino acid profiles

Function type:	Neutral Loss of 119.10 Da
Mass range:	m/z 150 to m/z 290
Cone Voltage:	30 V
Collision Energy:	25.0 eV
Collision gas pressure:	1.4 x 10^{-3} mbar Argon
Scan time:	3.0 secs (0.05 sec inter-scan delay)



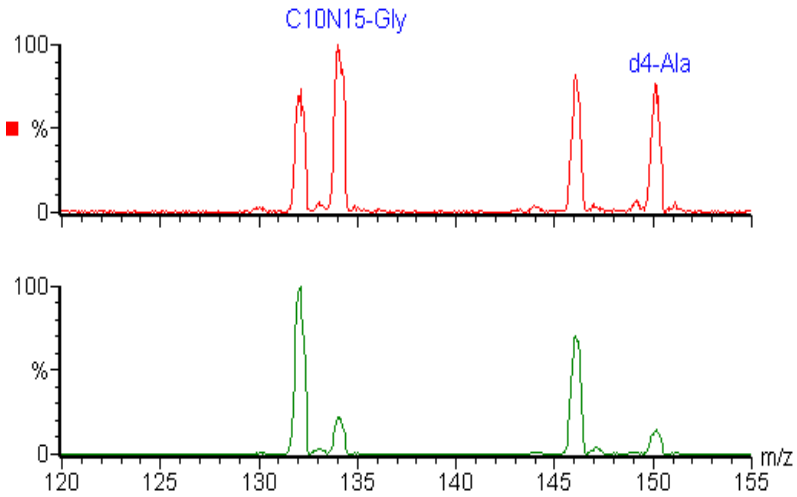
Normal basic amino acid profile from a healthy patient.



Normal patient*vs* patient with elevated levels of ciitrulline (*m/z* 232). This spectrum was acquired in the same experiment as shown in the elevated citrulline example above.

Alternative scan function for Glycine and Alanine

Function type:	Neutral Loss of 56.00 Da
Mass range:	m/z 120 to m/z 155
Cone Voltage:	25 V
Collision Energy:	8 eV
Collision gas pressure:	1.4 x 10^{-3} mbar Argon
Scan time:	0.4 secs (0.05 sec inter-scan delay)



Normal patient *vs* patient with elevated levels of glycine (*m/z* 132) and alanine (*m/z* 146). Note the presence of the analytes and the isotopically labelled internal standards used for quantification.

Appendix B Quantification

The use of multiple reaction monitoring (MRM) for the detection of metabolic disorders in neonatal bloodspots has been shown to be comparable to the use of full-scan data employed in a number of laboratories around the world. The use of MRM analysis allows for faster flow rates to be used for sample introduction, considerably reducing the inter-sample analysis time. In MRM analysis only specific metabolites may be monitored following validation of mass spectrometric methods for specific metabolites.

Multiple reaction monitoring is widely used in the pharmaceutical industry, especially during clinical trials, for the quantitative monitoring of drug metabolites. It seems logical to extend those methodologies to the problems encountered in neonatal screening, as the absolute levels of endogenous metabolites present in an abnormal sample are likely to be of use for the consulting physician. While the use of relative mass spectral peak intensity ratios yields useful information about the relative levels of endogenous metabolites to the spiked internal standards, this is not truly representative of the total amount of samples injected into the system. MRM data may be used to gain both absolute quantitative information, *via* integration of the individual chromatographic traces and relative quantitative information by simply combining the 'spectra' across a chromatographic peak and comparing peak intensity ratios.

The table below represents a summary of the different quantification methods available using the MassLynx and NeoLynx software suite. The MassLynx Quantify program uses classical chromatographic peak integration and area ratios to calculate the analyte concentrations. The individual analyte chromatogram traces are smoothed and integrated in order to determine the peak area used in subsequent calculations. NeoLynx uses a comparison of individual peak intensities, derived from combining and background subtracting the 'spectra' across the peak, rather than a true integration in time.

This comparison was effected using a series of eight MRM acquisitions in which a total of 52 species were detected. The data was then analyzed using the two different processes, and the results are presented in the Table below. The comparison column shows the absolute difference in the analyte/internal standard ratios and as a percentage of the ratio derived from the Quantify program. The average deviation and standard deviation were calculated for a total of eight separate acquisitions.

This comparative analysis to test the validity of the dual processing of the MRM data showed that the differences in the determined analyte/internal standard ratios only varied by a few percent between the two methods. Thus suggested that the use of combined data from an MRM acquisition may be used for elementary spectral analysis. As would be expected, the largest differences between the two methods is apparent when the peaks relating to the analytes are quite small - a significant number of the analytes measured are only present in minimal quantities in samples from healthy patients.

A comparison between the concentrations derived from MCA and MRM data is better illustrated in Example 4 in Appendix C.

Acylcarnitines

				FROM QUANTIFY		FROM NEOLYNX		COMPARISON		DEVIATION	
#	Name	IS#	Mass	Area	Ratio	Intensity	Ratio	d(ratio)	%(quan)	Ave %	SD
1	C2 carnitine	2	260.3	5620	1.324	7587	1.309	-0.015	-1.1	-0.48	1.55
2	d3-C2 carnitine	2	263.3	4245	1.000	5796	1.000				
3	C3 carnitine	4	274.3	1071	1.376	1445	1.389	0.013	1.2	1.48	1.55
4	d3-C3 carnitine	4	277.3	778	1.000	1040	1.000				
5	C4-carnitine	4	288.3	251	0.322	345	0.332	0.010	3.1	-0.90	3.72
6	C5-carnitine	4	302.3	364	0.468	495	0.476	0.008	1.7	-0.06	2.48
7	C6-carnitine	4	316.3	77	0.099	121	0.116	0.017	17.1	-0.43	13.60
8	C8:1-carnitine	10	342.4	387	0.130	496	0.123	-0.007	-5.4	-1.04	2.28
9	C8-carnitine	10	344.4	185	0.062	268	0.066	0.004	6.5	-0.39	6.71
10	d3-C8-carnitine	10	347.4	2971	1.000	4039	1.000				
11	C10:1-carnitine	10	370.4	236	0.079	327	0.081	0.002	2.5	3.20	8.63
12	C10-carnitine	10	372.4	314	0.106	418	0.103	-0.003	-2.8	-3.65	2.26
13	C4DC-carnitine	10	374.4	1042	0.351	2486	0.615	0.264	75.2	83.54	10.19
14	C5DC-carnitine	10	388.4	132	0.044	148	0.037	-0.007	-15.9	24.18	42.57
15	C12-carnitine	10	400.4	170	0.057	229	0.057	0.000	0.0	-6.19	13.60
16	C14:2-carnitine	22	424.5	159	0.036	212	0.035	-0.001	-2.8	1.90	18.39
17	C14:1-carnitine	22	426.5	151	0.035	172	0.029	-0.006	-17.1	-0.48	11.67
18	C14-carnitine	22	428.5	217	0.050	252	0.042	-0.008	-16.0	-3.10	5.86
19	C14-Ohcarnitine	22	444.5	22	0.005	44	0.007	0.002	40.0	-3.93	33.71
20	C16:1carnitine	22	454.5	126	0.029	175	0.029	0.000	0.0	0.54	7.68
21	C16-carnitine	22	456.5	1712	0.391	2321	0.385	-0.006	-1.5	0.34	2.04
22	d3-C16-carnitine	22	459.5	4375	1.000	6030	1.000				
23	C16-OH-carnitine	22	472.5	60	0.014	99	0.016	0.002	14.3	4.75	55.04
24	C18:2-carnitine	22	480.5	974	0.223	1307	0.217	-0.006	-2.7	-1.37	2.39
25	C18:1-carnitine	22	482.5	4215	0.963	5731	0.950	-0.013	-1.3	0.25	1.88
26	C18-carnitine	22	484.5	1465	0.335	1977	0.328	-0.007	-2.1	-0.45	1.80
27	C18:1-OH-carnitine	22	498.5	69	0.016	94	0.016	0.000	0.0	-0.16	9.83

Amino Acids

			FROM QUANTIFY			FROM NEOLYNX		COMPARISON		DEVIATION	
#	Name	IS#	Mass	Area	Ratio	Intensity	Ratio	d(ratio)	%(quan)	Ave %	SD
1	Glycine	2	132.3	40	0.097	79	0.142	0.045	46.4	25.83	37.01
2	13C-15N-Glycine	2	134.3	407	1.000	555	1.000				
3	Alanine	4	146.3	8064	3.690	11170	3.657	-0.033	-0.9	-0.49	2.10
4	d4-Alanine	4	150.3	2186	1.000	3054	1.000				
5	Valine	6	174.3	9099	2.522	12620	2.553	0.031	1.2	1.45	1.50
6	d8-Valine	6	182.3	3607	1.000	4944	1.000				
7	Glutamine	9	186.3	9582	0.587	13990	0.618	0.031	5.3	4.24	2.58
8	Leucine+Isoleucine	9	188.3	34140	2.093	47520	2.099	0.006	0.3	-0.04	0.74
9	d3-Leucine	9	191.3	16311	1.000	22630	1.000				
10	Methionine	11	206.3	3137	0.285	4426	0.288	0.003	1.1	1.19	2.62
11	d3-Methionine	11	209.3	10996	1.000	15360	1.000				
12	Citrulline	11	215.3	2662	0.242	3624	0.236	-0.006	-2.5	-2.31	1.58
13	Phenylalanine	14	222.3	27248	0.828	37980	0.833	0.005	0.6	-0.01	0.78
14	d5-Phenylalanine	14	227.3	32897	1.000	45600	1.000				
15	Tyrosine	16	238.3	19266	1.281	26490	1.284	0.003	0.2	-0.31	1.35
16	d4-Tyrosine	16	242.3	15043	1.000	20630	1.000				
17	Aspartate	19	246.3	16509	1.753	22930	1.768	0.015	0.9	0.36	1.30
18	Glutamate	19	260.3	16340	1.735	22430	1.729	-0.006	-0.3	0.14	1.39
19	d3-Glutamate	19	263.3	9418	1.000	12970	1.000				

Appendix C Examples of Data Processing Using NeoLynx

Data Files Supplied

Standard01-Standard10	For Example 1
Blood01-Blood10	For Example 2
Sample01-Sample08	For Example 3
MCA Test 01 - MCA Test 11	For Example 4
MRM Test 01 - MRM Test 11	For Example 4

Before processing each batch, check that the processing parameters are appropriate for the type of data you are analyzing. The user is expected to prepare the Sample Lists in an appropriate format, and to prepare appropriate NeoNatal Rule sets.

Information relating to the appropriate mass / compound relationship may be found elsewhere in the NeoLynx Research Manual.

The data provided for this exercise is intended for tutorial purposes only, and no absolute information may be inferred from the profiles observed.

EXAMPLE 1: CHECK PEAK INTENSITIES TO VERIFY PRESENCE OF A COMPOUND

Objective: The objective of this exercise is to automatically analyze the spectra provided and to determine which compounds are not present (and thus by default, which compounds are present) from the list provided.

The samples STANDARD01-STANDARD10 were mixtures of standard compounds that had been butylated and reconstituted in 50% aqueous acetonitrile.

The samples were analyzed using a five-function MCA acquisition:

1) Parents of *m/z* 85 for acylcarnitines

2) Neutral loss of 102 Da for neutral and acidic amino acids

3) Neutral loss of 119 Da for basic amino acids

4) Neutral loss of 56 Da for glycine and alanine

5) Neutral loss of 161 Da for arginine

The standard compounds used for this experiment were:

Free carnitine, Phenylalanine, Methionine, Leucine, Ornithine, Citrulline, Glycine and Arginine

- Manually check the spectra and decide on appropriate criteria for the presence or absence of each compound in question.

- Using NeoLynx, automatically process the data to check for minimum peak intensities (Rule 0). Examine the results for the 10 Sample files, and record which compounds are present in each mixture.

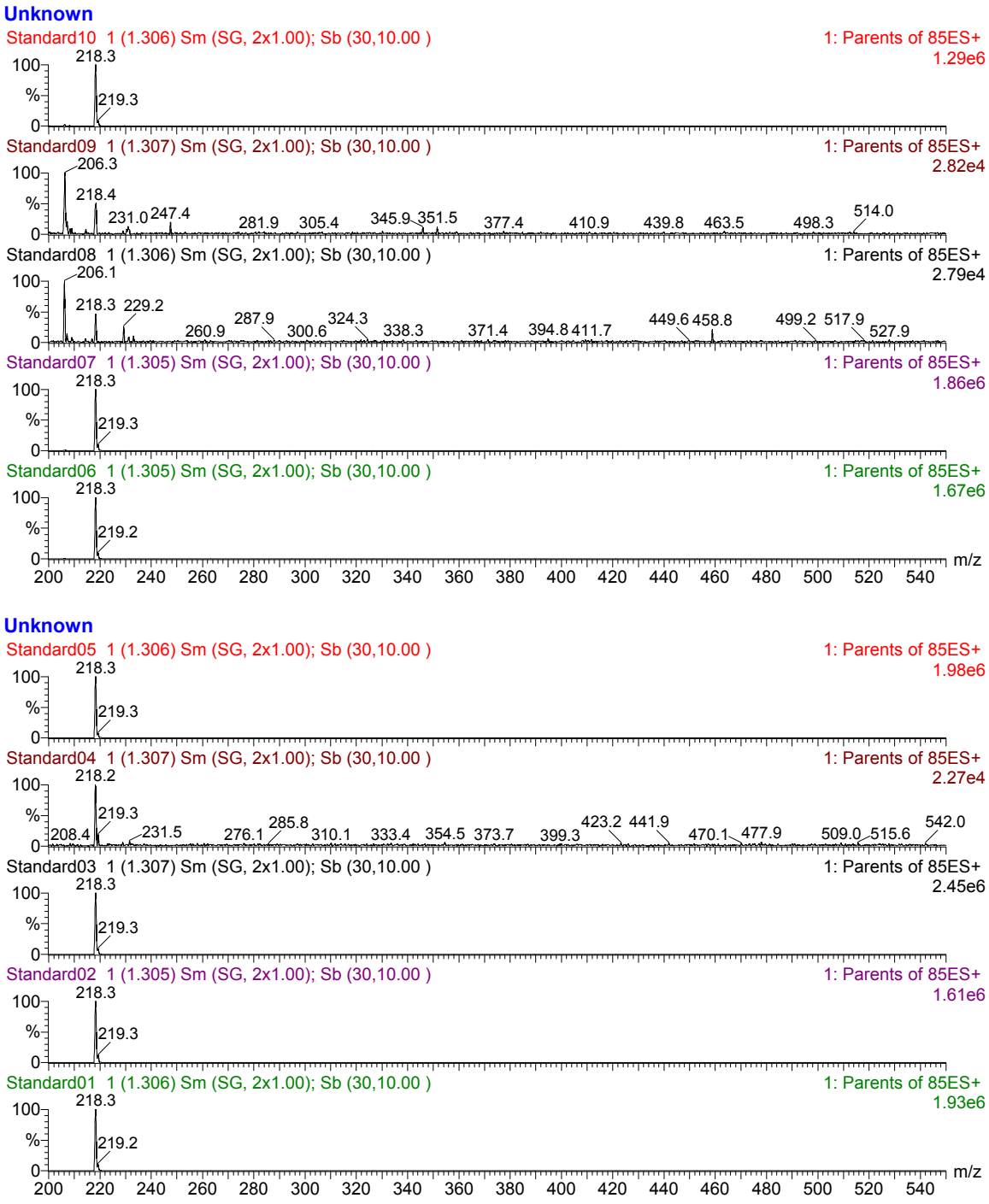
EXAMPLE 1: WORKING TABLE

Test Name	Rule	Func	Min Ratio	Max Ratio	Mass 1	Mass 2	Msg Low	Msg High	Msg OK	IS Conc

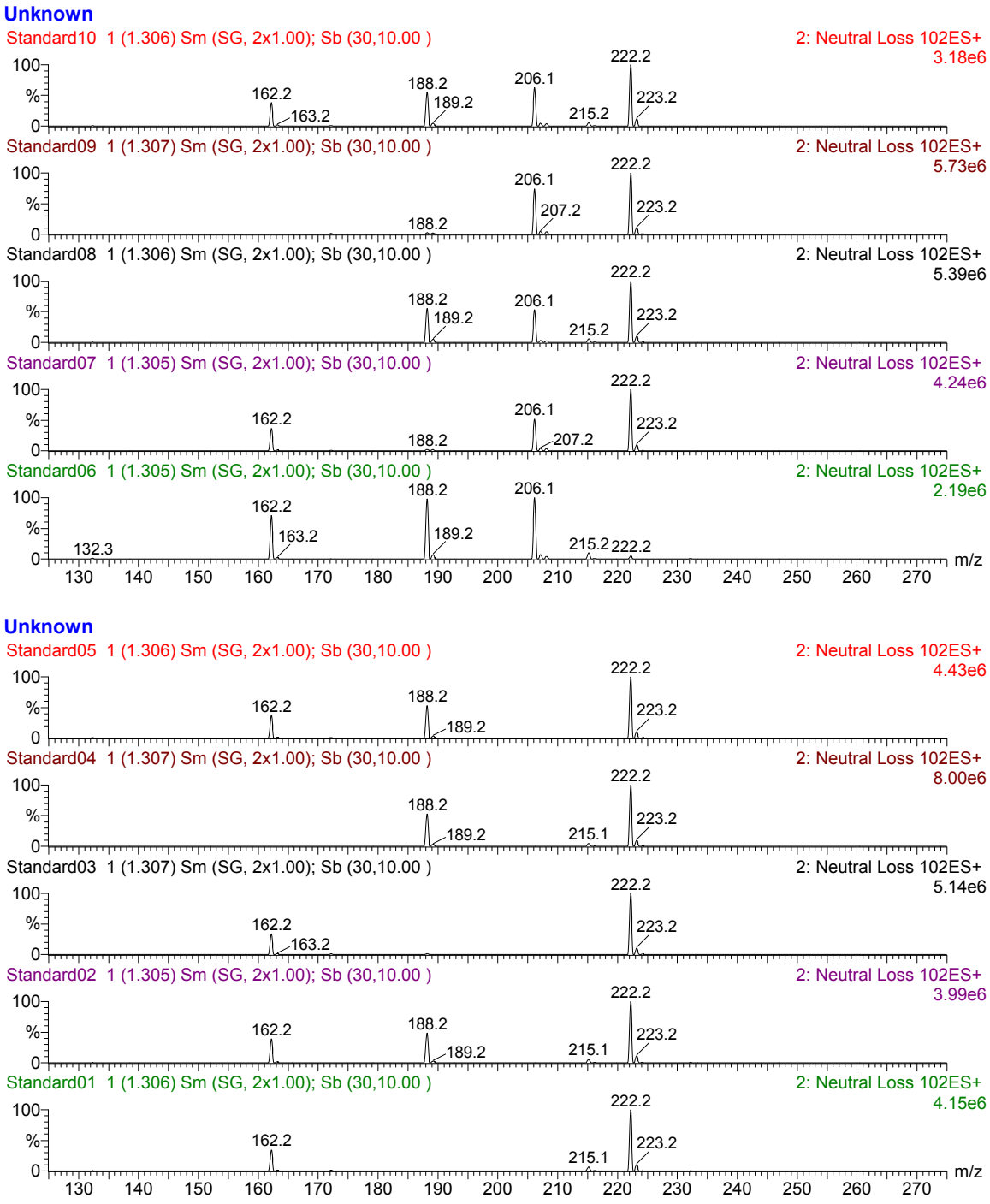
EXAMPLE 1: CONCLUSIONS

SAMPLE	COMPOUNDS PRESENT
Standard 01	
Standard 02	
Standard 03	
Standard 04	
Standard 05	
Standard 06	
Standard 07	
Standard 08	
Standard 09	
Standard 10	

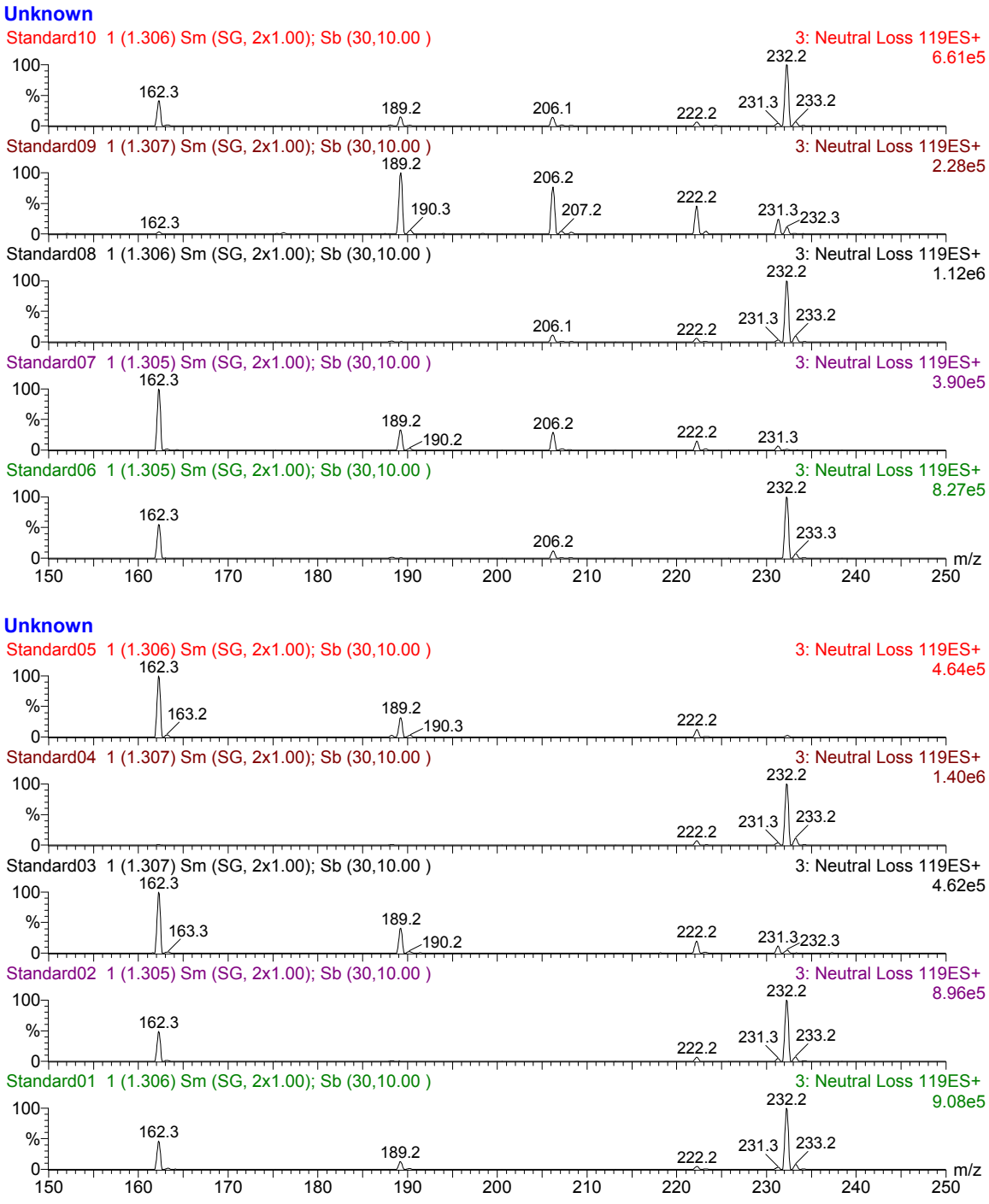
EXAMPLE 1: Function 1



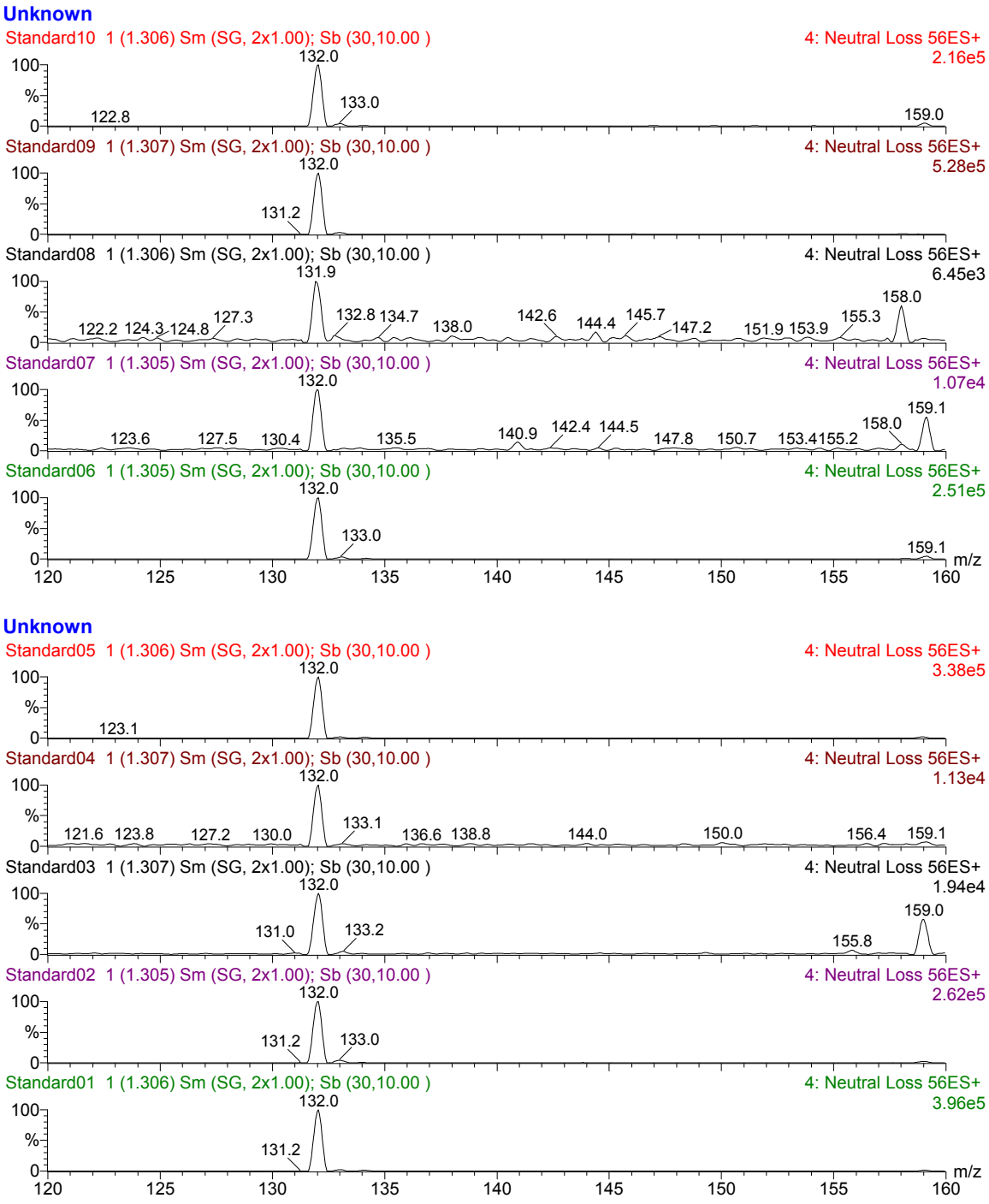
EXAMPLE 1: Function 2



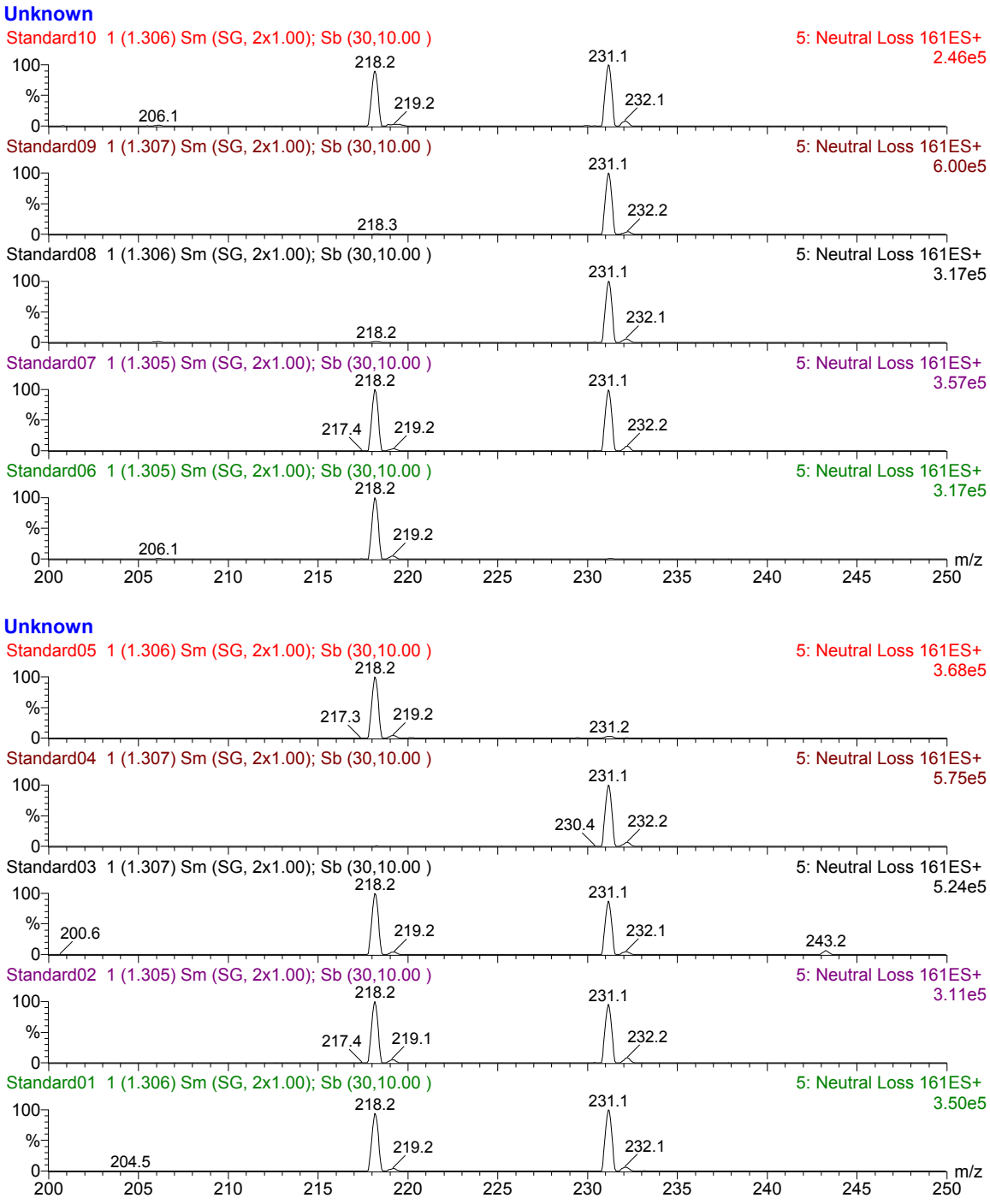
EXAMPLE 1: Function 3



EXAMPLE 1: Function 4



EXAMPLE 1: Function 5



EXAMPLE 2: EXAMINE SPECTRA FROM SPIKED BLOOD SPOTS

Objective: The purpose of this exercise is to prepare a rule table to automatically detect which compounds have been spiked into the blood samples.

The samples BLOOD01 to BLOOD10 were prepared and analyzed. A standard dried blood sample paper was divided into 10 separate pieces, and some were spiked with methanolic solutions of pure amino acids. The samples were then extracted and derivatized in the normal way.

The samples were analyzed in a five-function MCA acquisition:

1) Parents of *m/z* 85 for acylcarnitines

2) Neutral loss of 102 Da for neutral and acidic amino acids

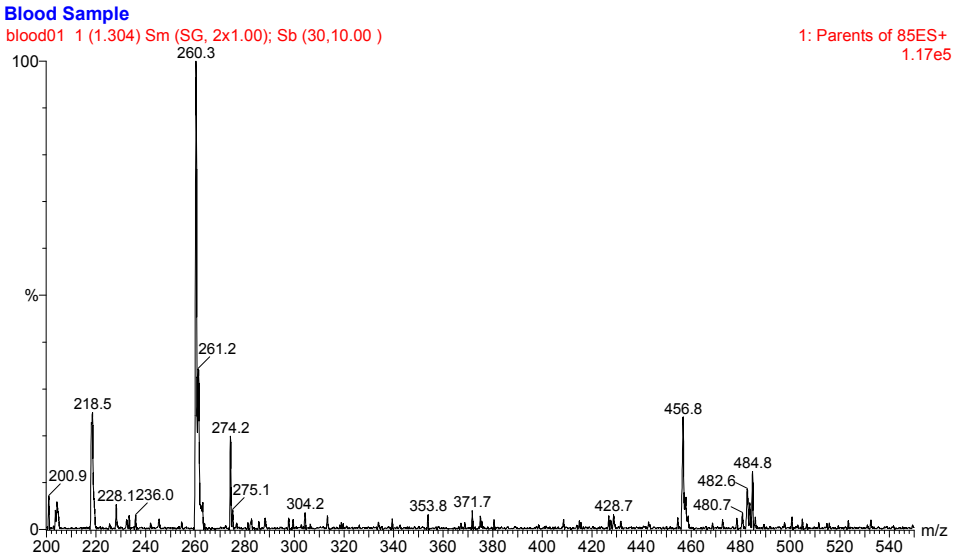
3) Neutral loss of 119 Da for basic amino acids

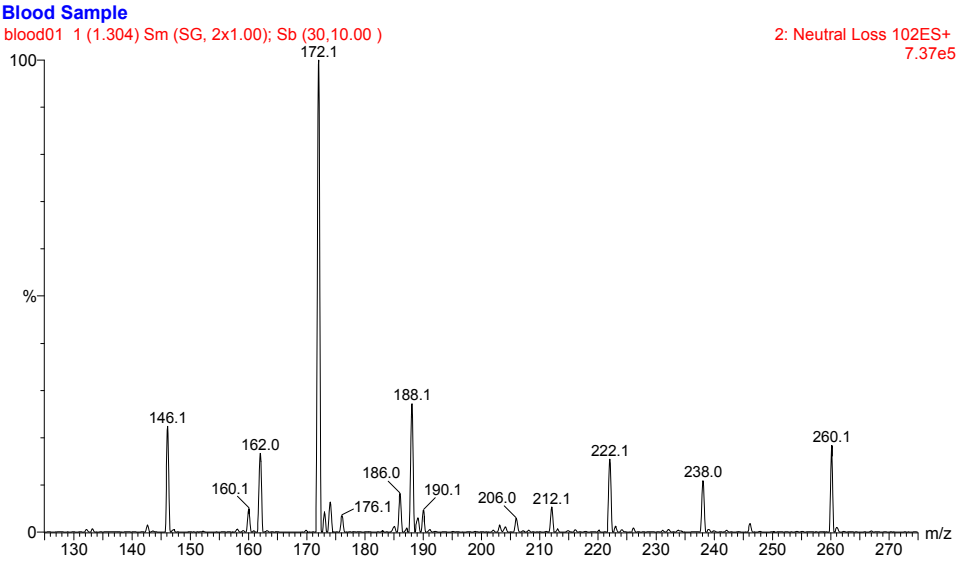
4) Neutral loss of 56 Da for glycine and alanine

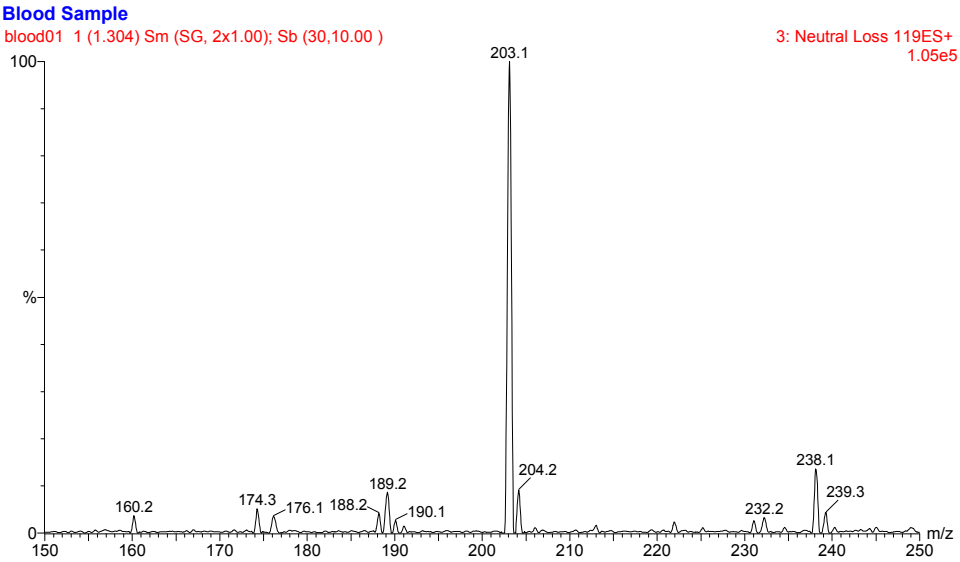
5) Neutral loss of 161 Da for arginine

- BLOOD01data is from unadulterated blood.
- Examine the spectra to see what has been added to the blood sample.
- Using the simple ratio method (Rule 1), ratio the following compounds to the C2 carnitine (m/z 260), Proline (m/z 172) and Glutamine (m/z 203) peaks in functions 1 to 3, respectively: Carnitine, Phe, Leu, Met, Orn, Cit. Normal ratios may be calculated from the sample Blood01 which has not been altered. Abnormal levels were arbitrarily set at 3 times the normal peak intensity ratio for inclusion as the Maximum Threshold in the Neonatal rule table.
- Which samples have been adulterated, and with what?
- Report the abnormal ratios.

EXAMPLE 2: BLOOD 01







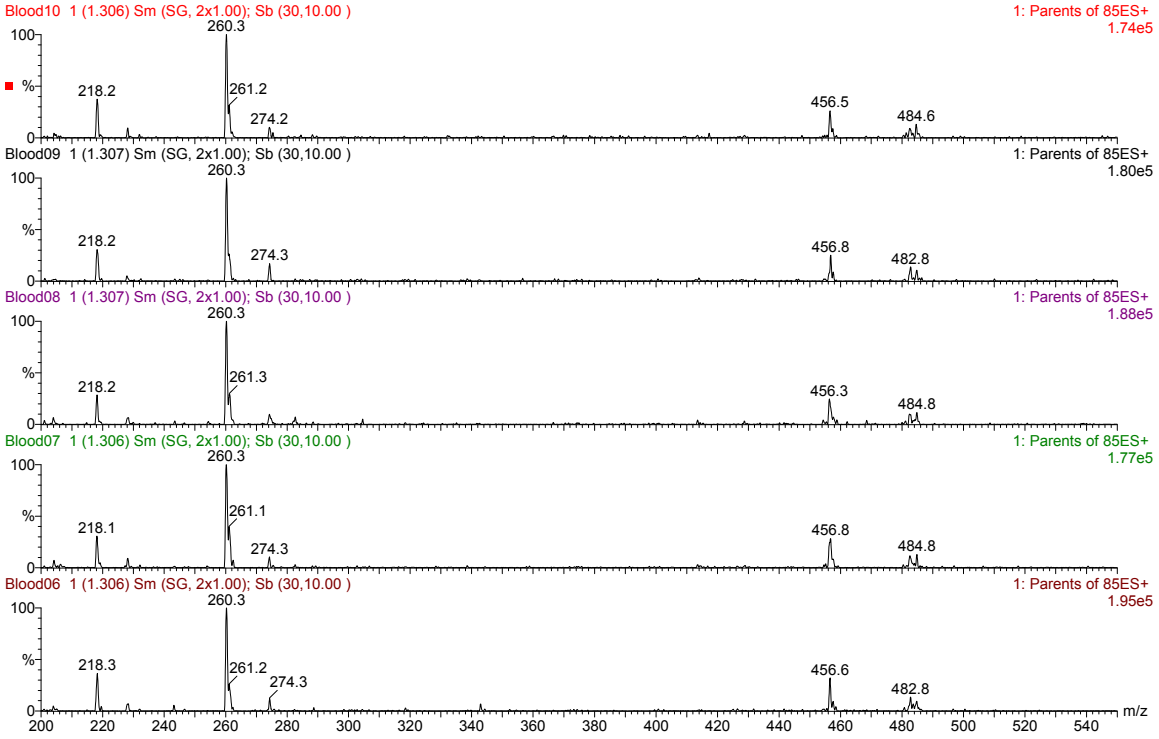
EXAMPLE 2: WORKING TABLE

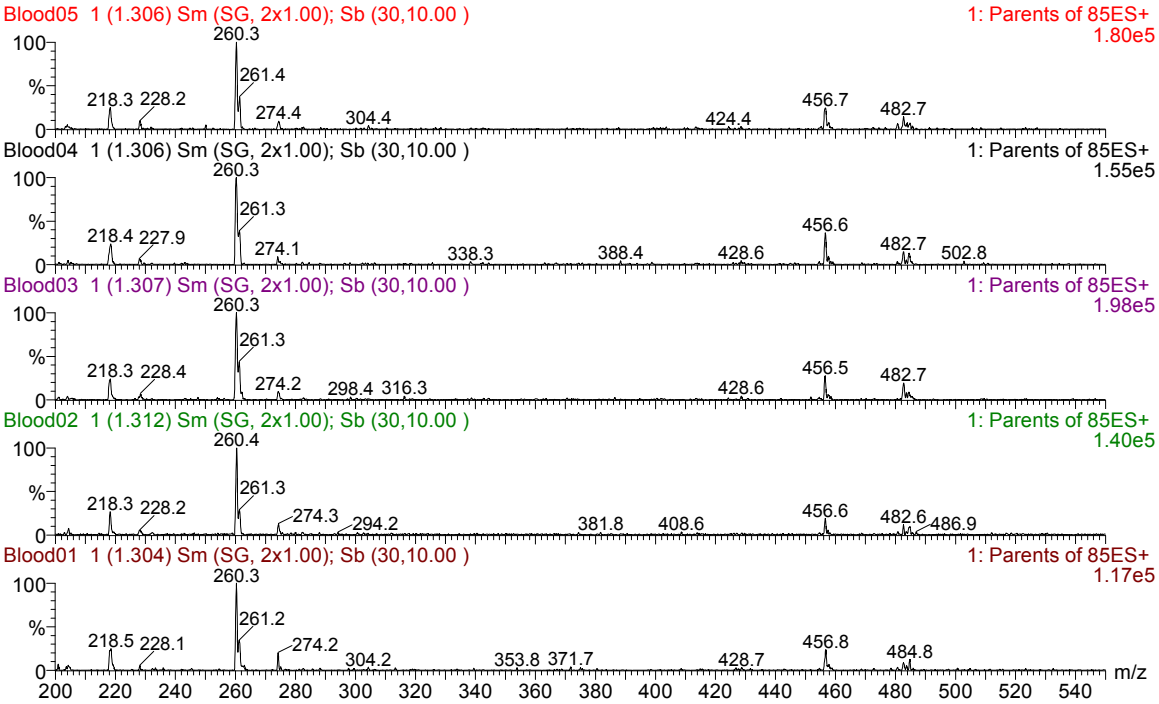
Test	Rule	Func	Min Ratio	Max Ratio	Mass 1	Mass 2	Msg Low	Msg High	Msg OK	IS Conc

EXAMPLE 2: CONCLUSIONS

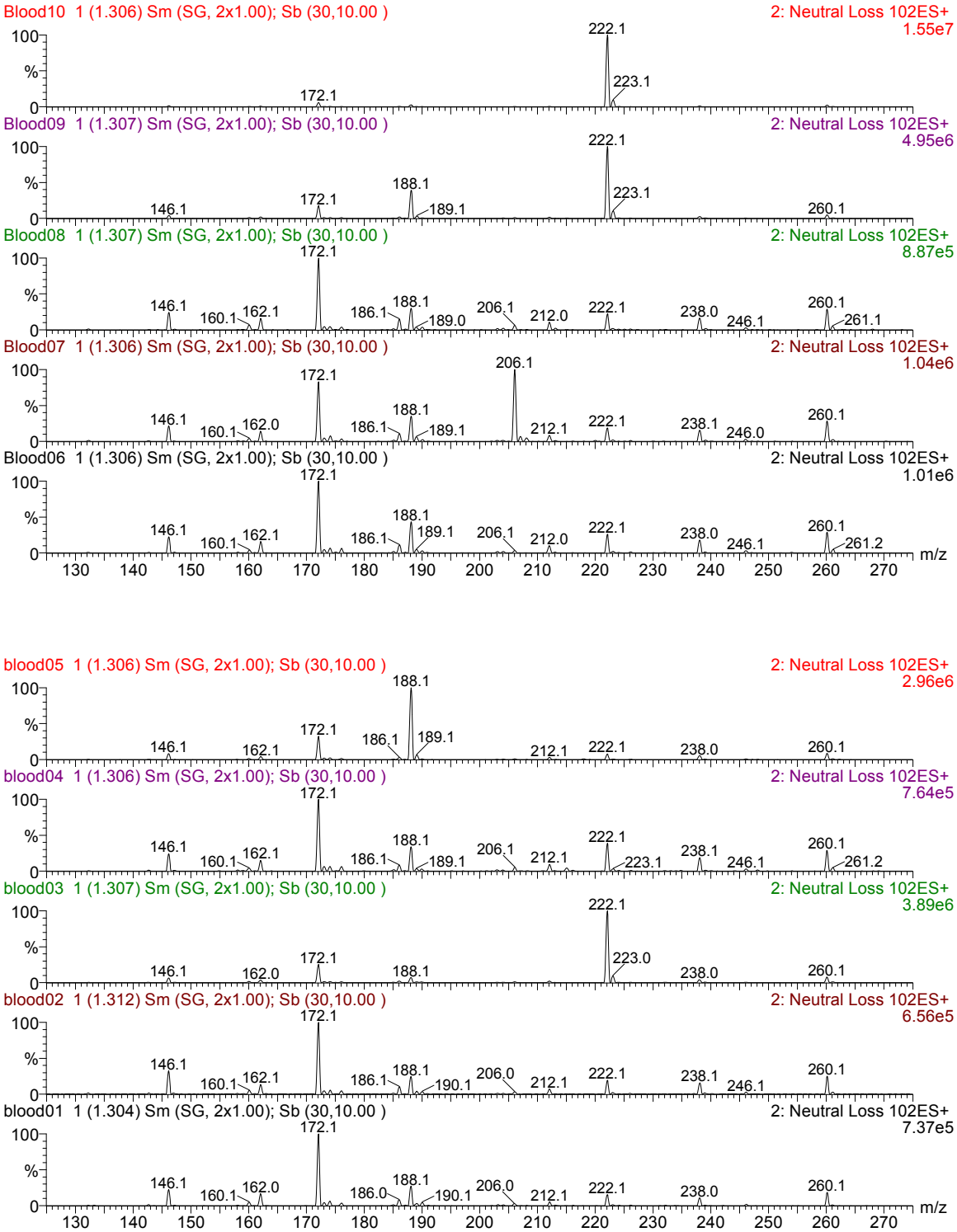
SAMPLE	COMPOUNDS ADDED (AMOUNT)
Blood 01	
Blood 02	
Blood 03	
Blood 04	
Blood 05	
Blood 06	
Blood 07	
Blood 08	
Blood 09	
Blood 10	

EXAMPLE 2: Function 1

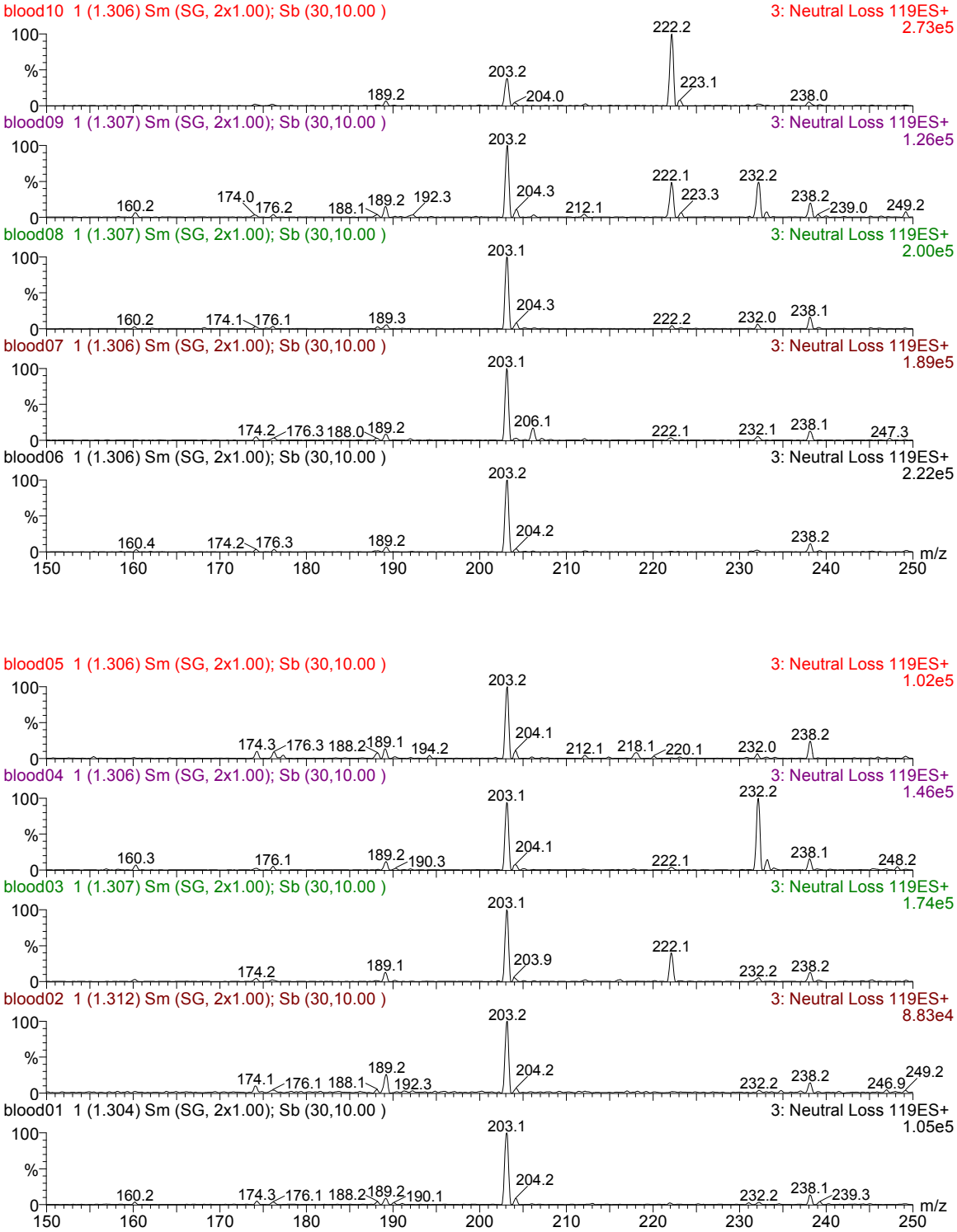




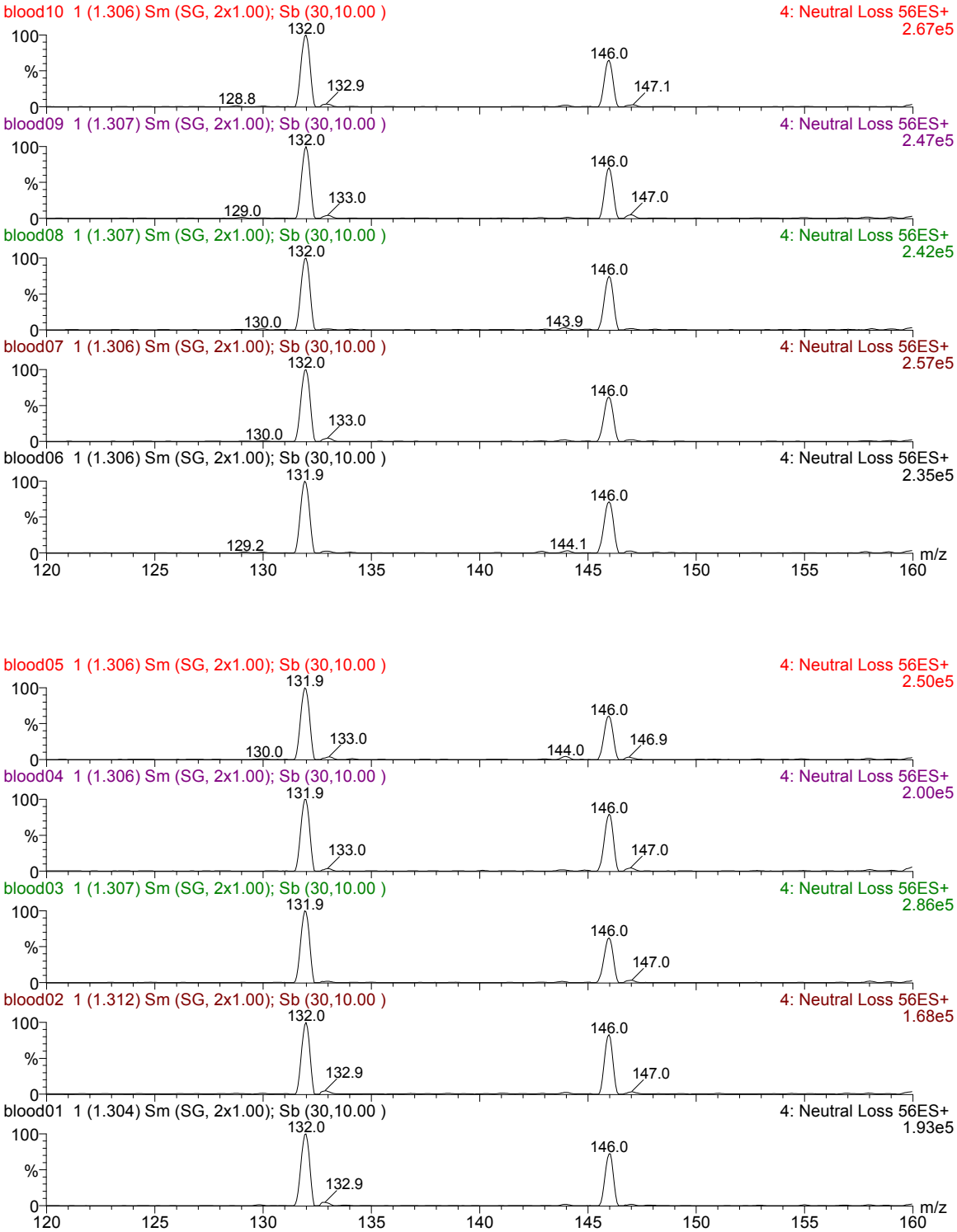
EXAMPLE 2: Function 2



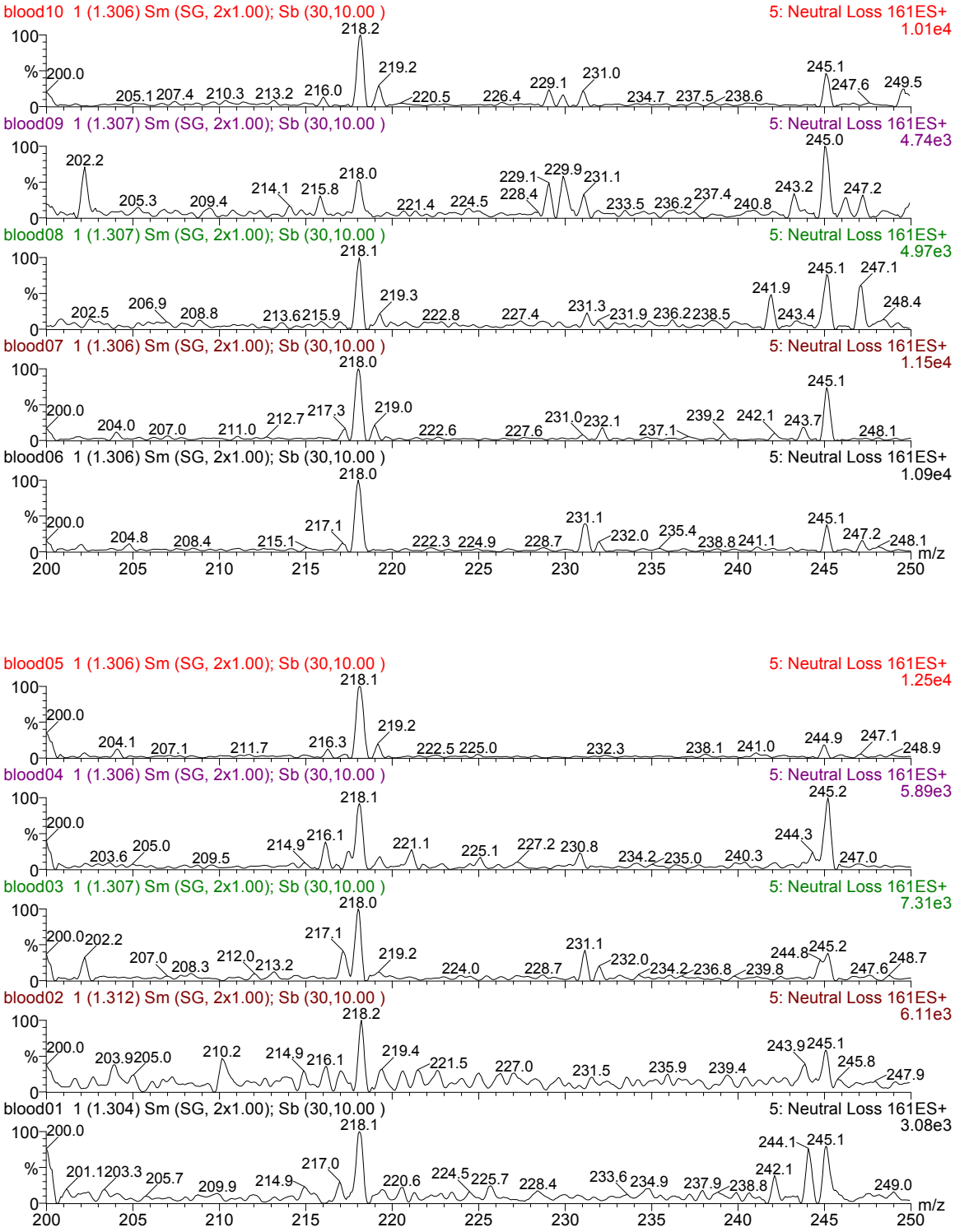
EXAMPLE 2: Function 3



EXAMPLE 2: Function 4



EXAMPLE 2: Function 5



EXAMPLE 3: PROCESS SAMPLES CONTAINING INTERNAL STANDARDS TO YIELD QUANTIFIED RESULTS

Objective: The purpose of this exercise is to quantify some metabolites of interest in eight patient samples from information given about the concentration of internal standards.

Samples SAMPLE01 to SAMPLE08 were prepared from affected neonatal blood spots and were acquired using isotopically labeled internal standards, the concentrations of which are known. Process the 8 data files NEO01 to NEO08 given the following information:

The samples were analyzed using a two-function MCA acquisition:

1) Parents of *m/z* 85 for acylcarnitines

2) Neutral loss of 102 Da for neutral and acidic amino acids

The following internal standards and concentrations were used:

d3-Free carnitine	10µM/L
d3-C2-carnitine	10uM/L
d3-C8-carnitine	10uM/L
d3-C16-carnitine	10uM/L
d5-phenylalanine	200uM/L
d3-leucine	400uM/L
d3-methionine	150uM/L
d4-tyrosine	400uM/L

Normally, the most accurate quantification is achieved if each analyte has it's own isotopically labeled internal standard. This is not financially viable for acylcarnitines, so the following approximation may be used:

Use d3-C2-carnitine as the internal standard for short-chain (C2-C5) acylcarnitines.

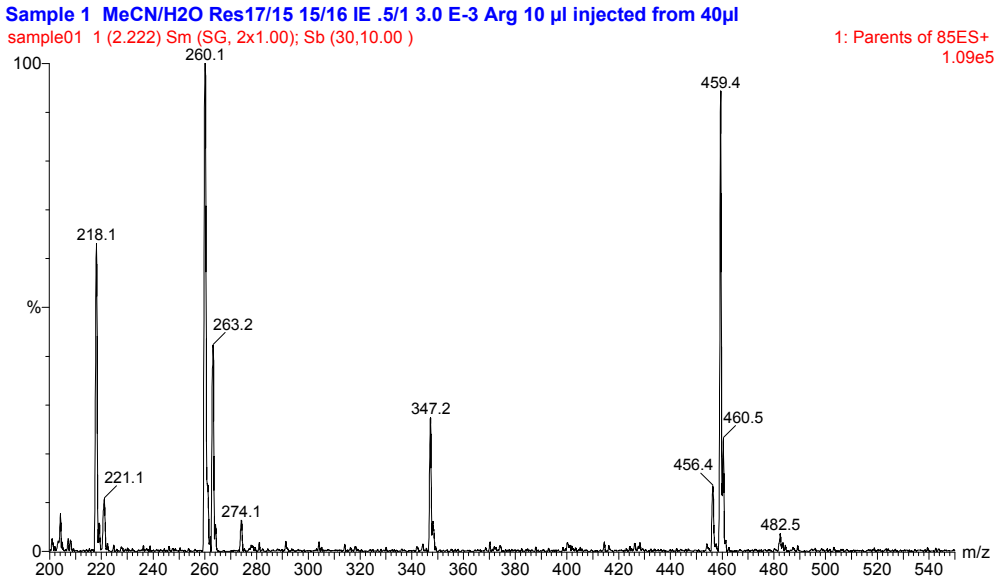
Use d3-C8 carnitine as the internal standard for the medium-chain (C6-C12) acylcarnitines.

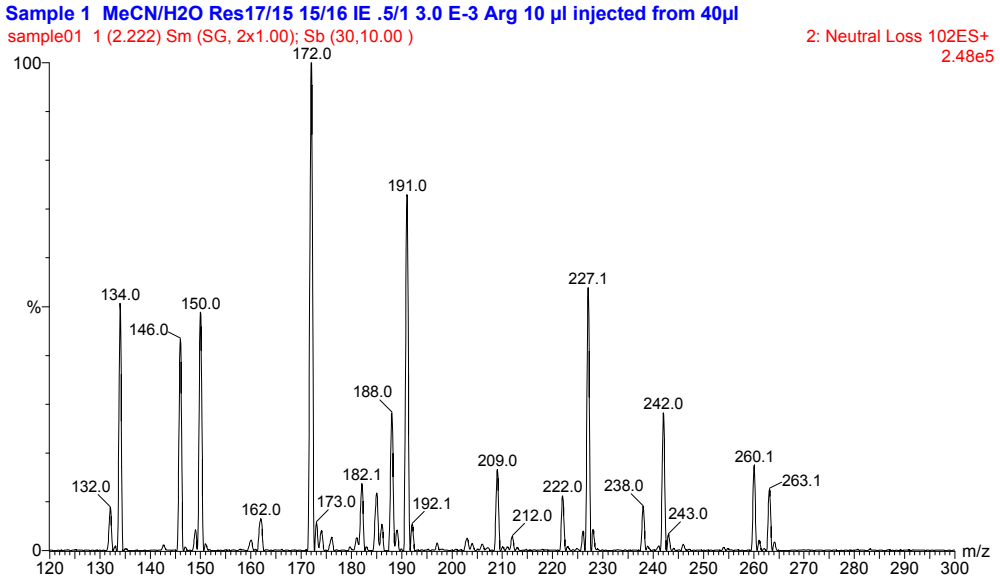
Use d3-C16 carnitine as the internal standard for the long-chain (>C12) acylcarnitines.

The sample in NEO01 is normal.

- Examine the spectra manually.
- Determine the appropriate peak intensity ratios for the compounds and internal standards of interest for the normal samples. Note the internal standards have been added to each sample at the nominal cut-off level for the amino acids, and at 10uM/L for the acylcarnitines. Use discretion in determining acylcarnitine cut-off levels.
- Use NeoLynx to calculate the concentrations of the acylcarnitines and amino acids in the abnormal samples.
- Determine the [Phe]/[Tyr] concentration ratio automatically for the abnormal samples.

EXAMPLE 3: SAMPLE 01





EXAMPLE 3: WORKING TABLE

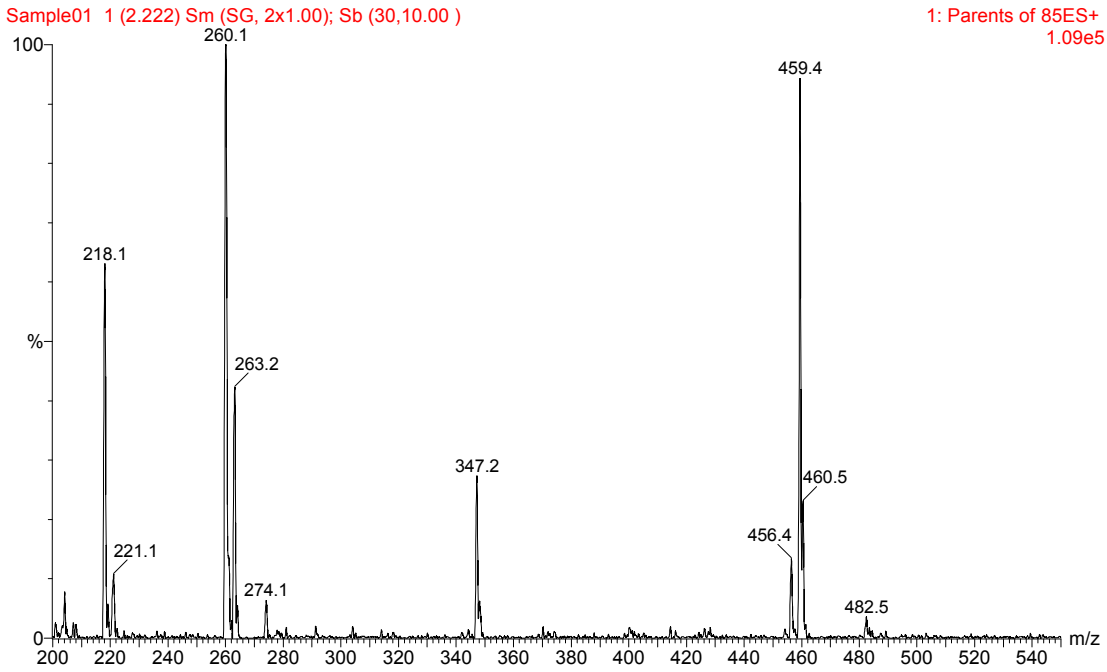
Test	Rule	Func	Min Ratio	Max Ratio	Mass 1	Mass 2	Msg Low	Msg High	Msg OK	IS Conc

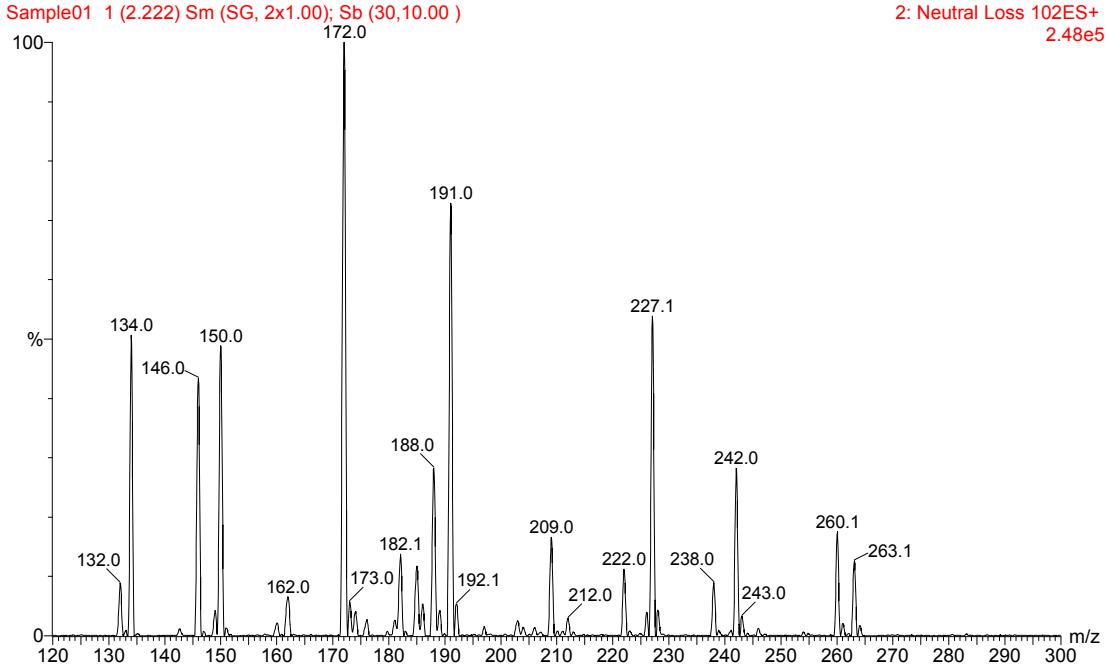
EXAMPLE 3: CONCLUSIONS

SAMPLE	ABNORMAL MASSES / COMPOUND PRESENT (CONCENTRATION) - INFERENCE
Sample 01	
Sample 02	
Sample 03	
Sample 04	
Sample 05	
Sample 06	
Sample 07	
Sample 08	

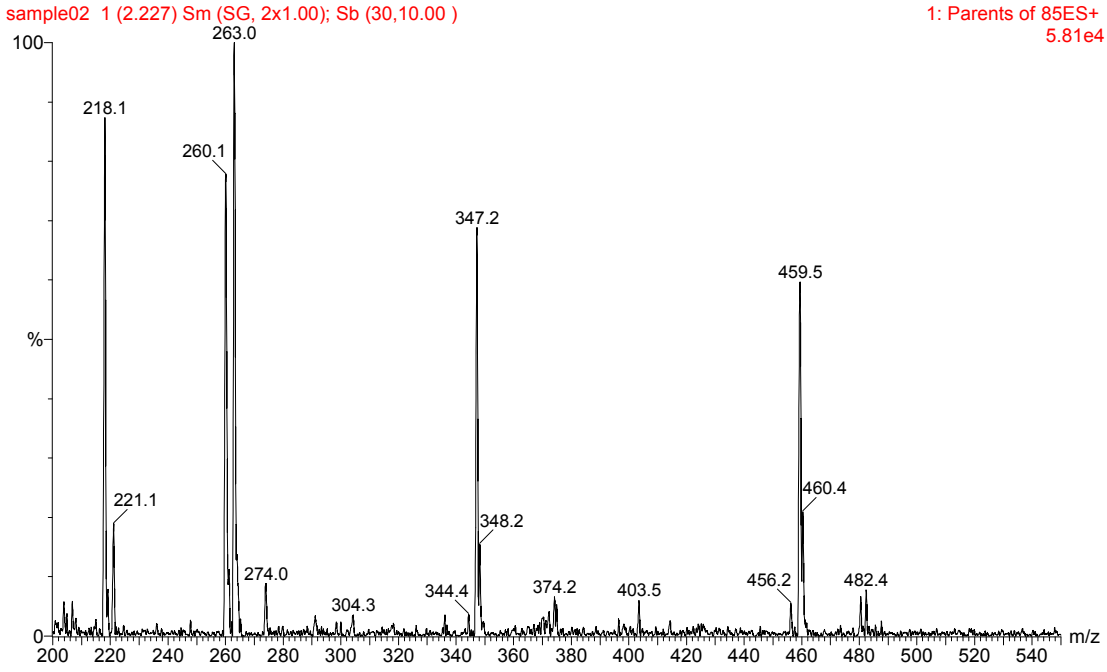
Manual Examination of Spectra for Example 3

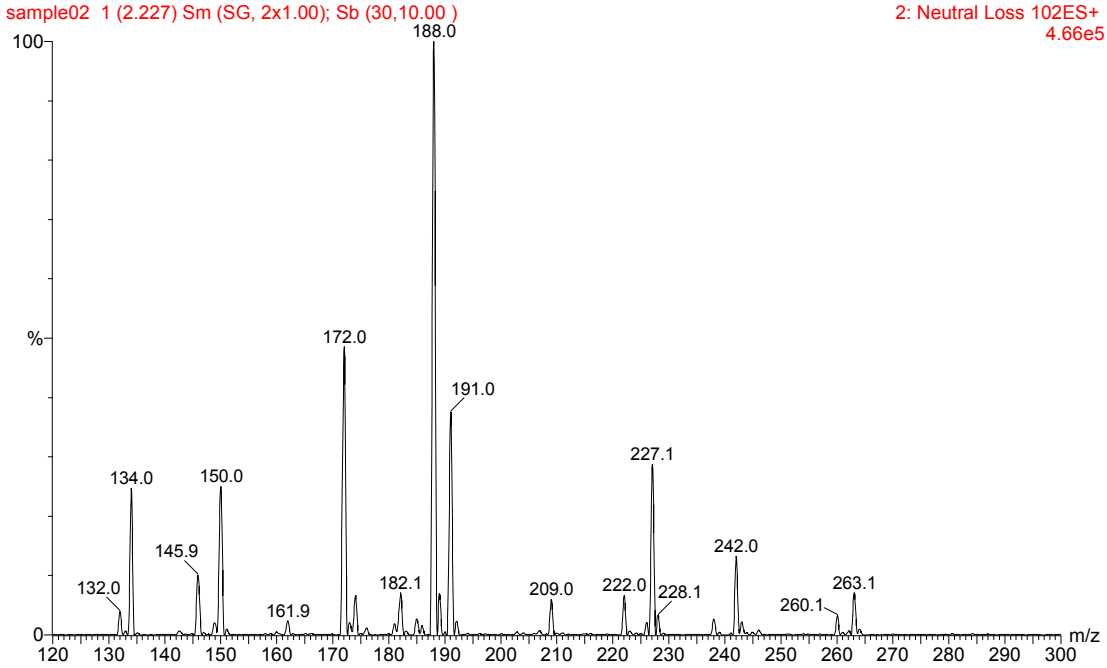
EXAMPLE 3: Spectra for Sample 01



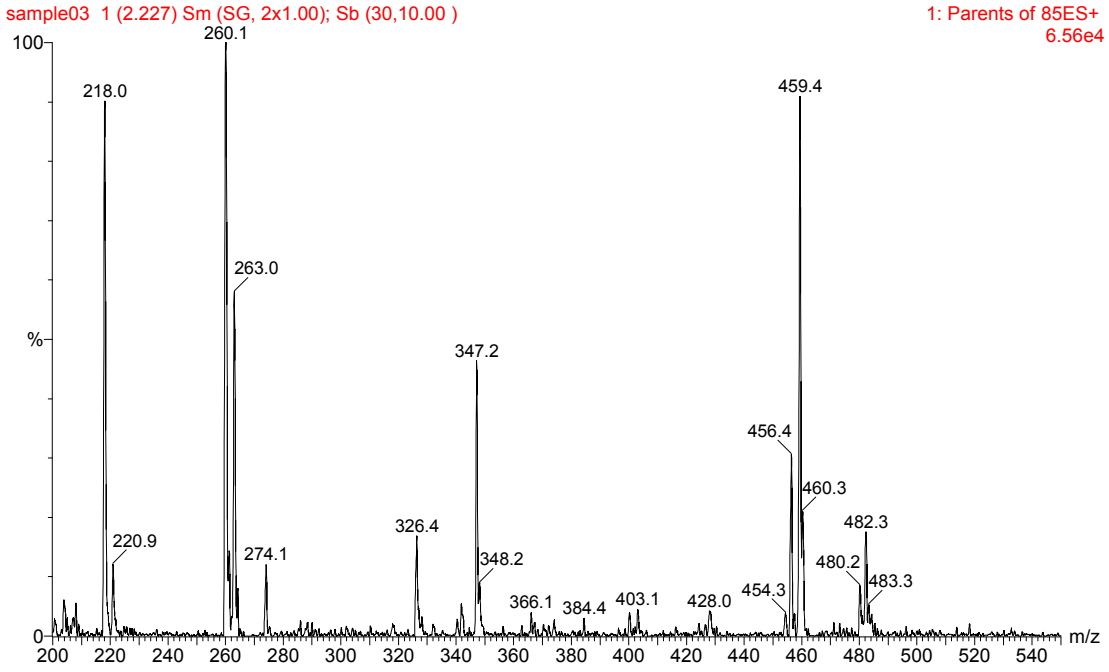


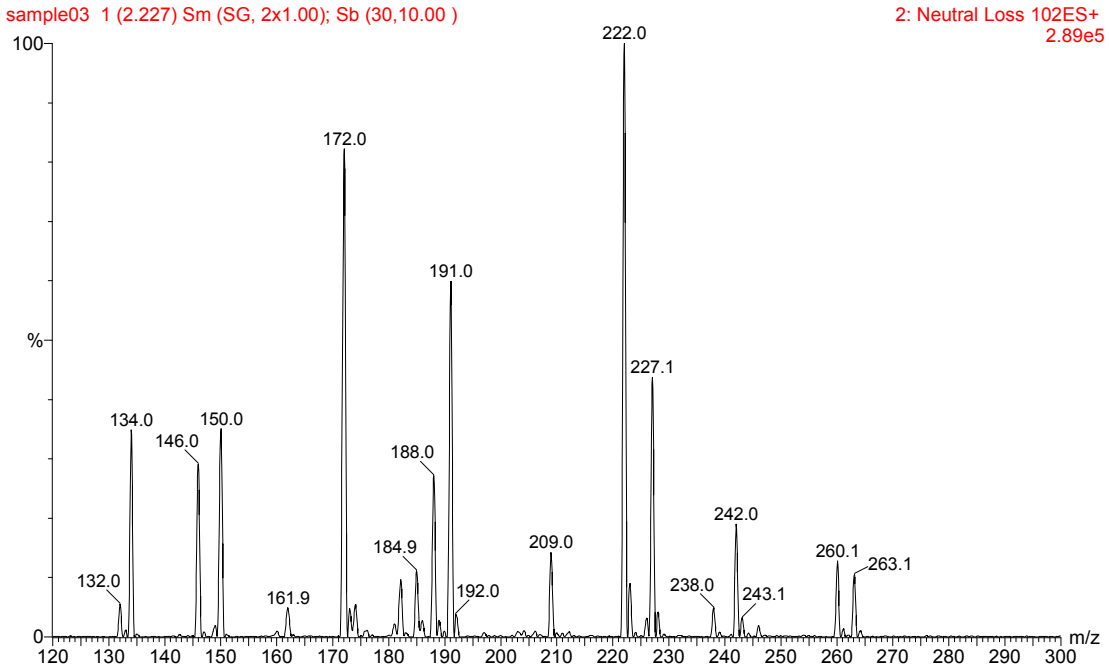
EXAMPLE 3: Spectra for Sample 02



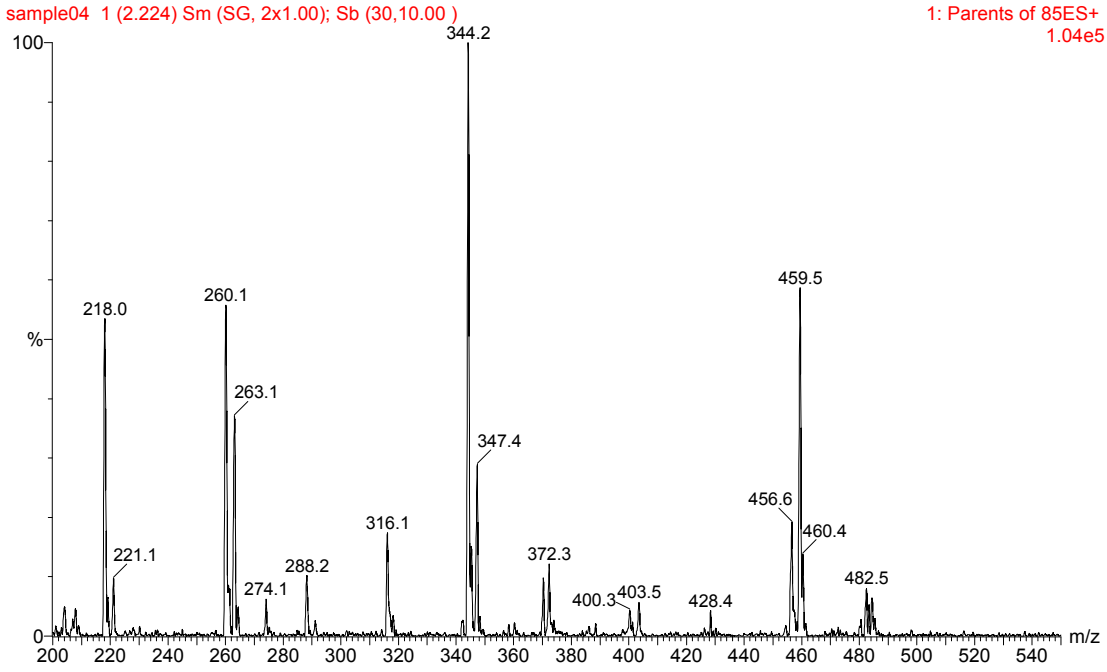


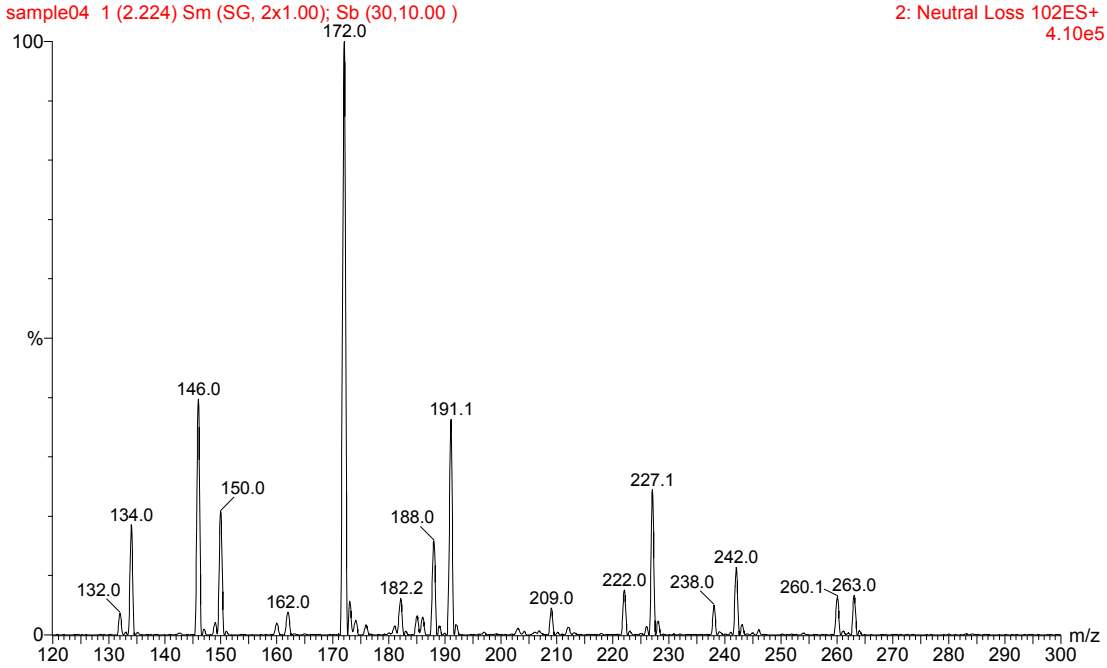
EXAMPLE 3: Spectra for Sample 03



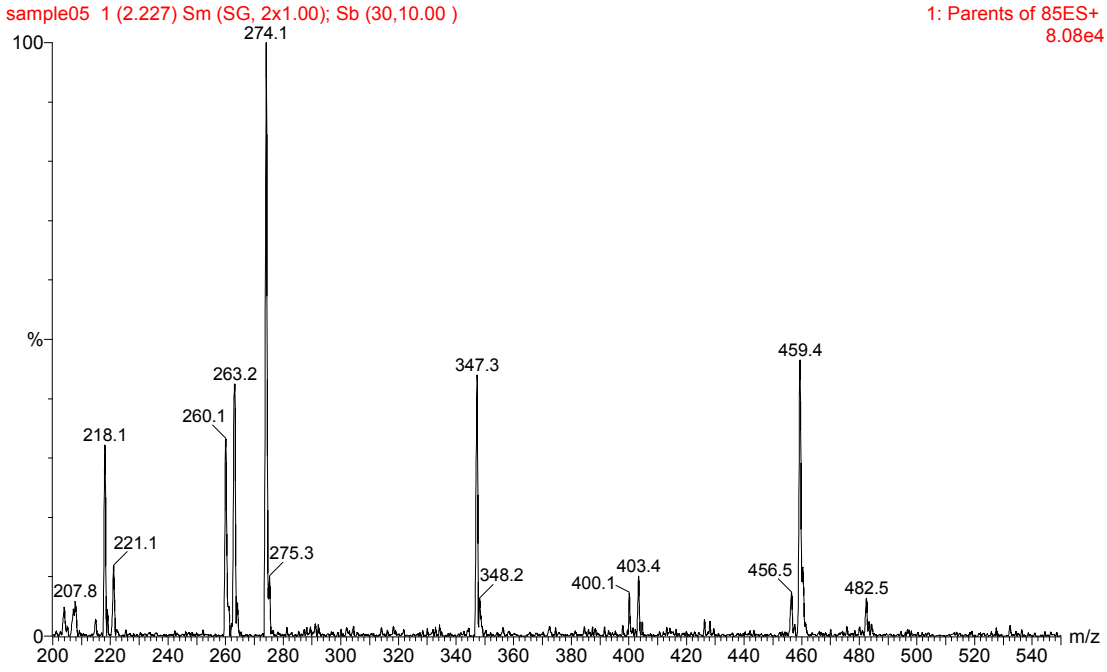


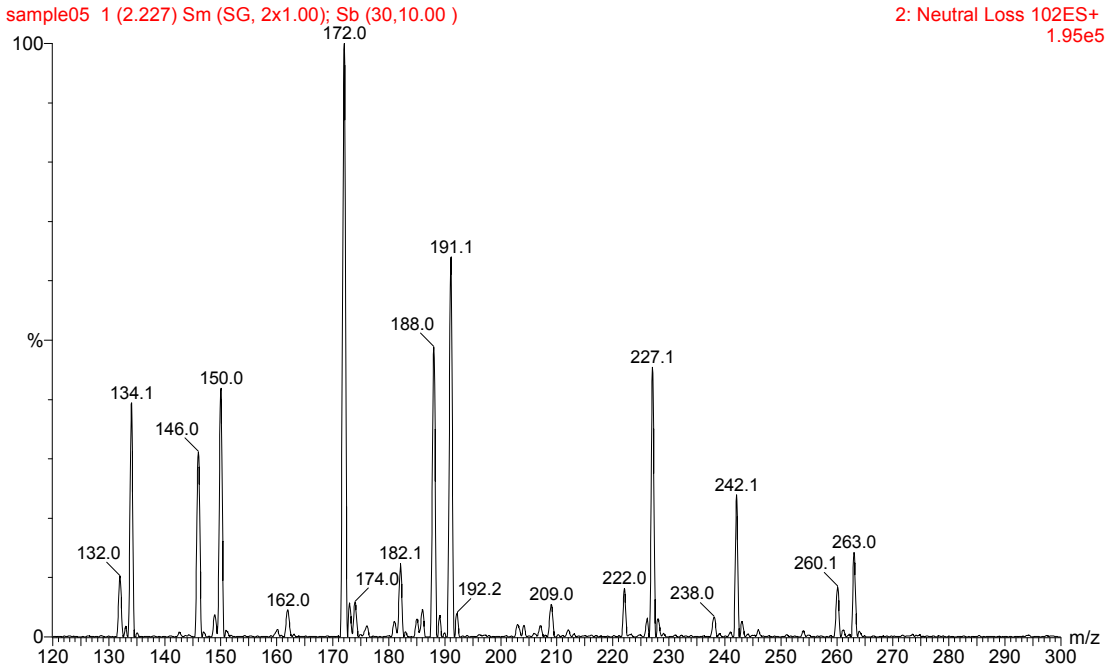
EXAMPLE 3: Spectra for Sample 04



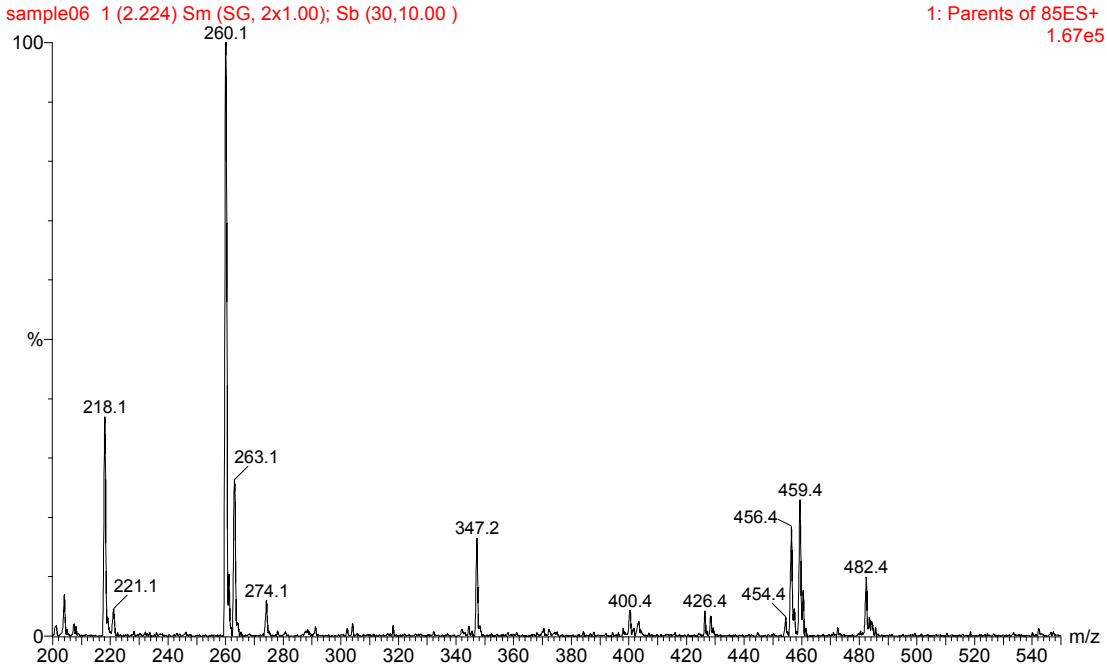


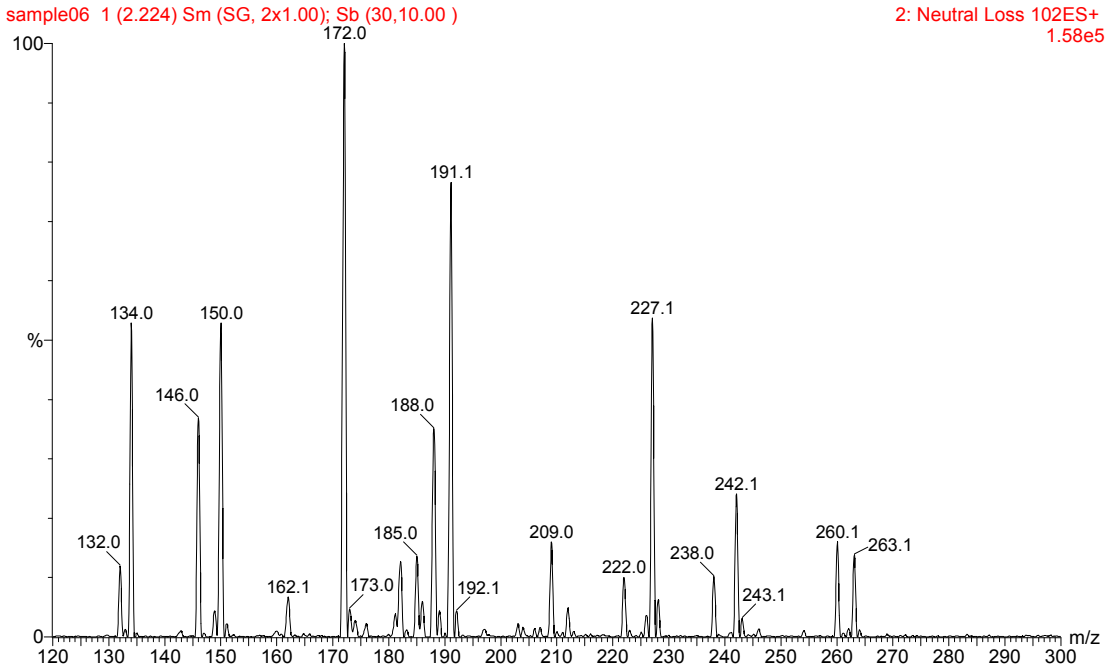
EXAMPLE 3: Spectra for Sample 05



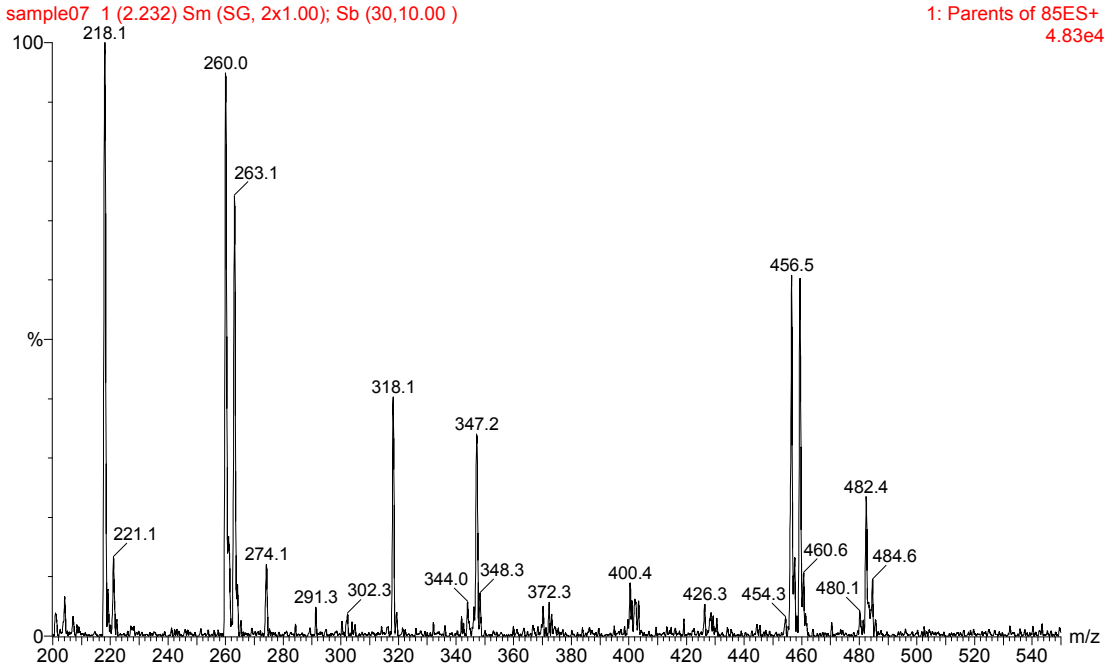


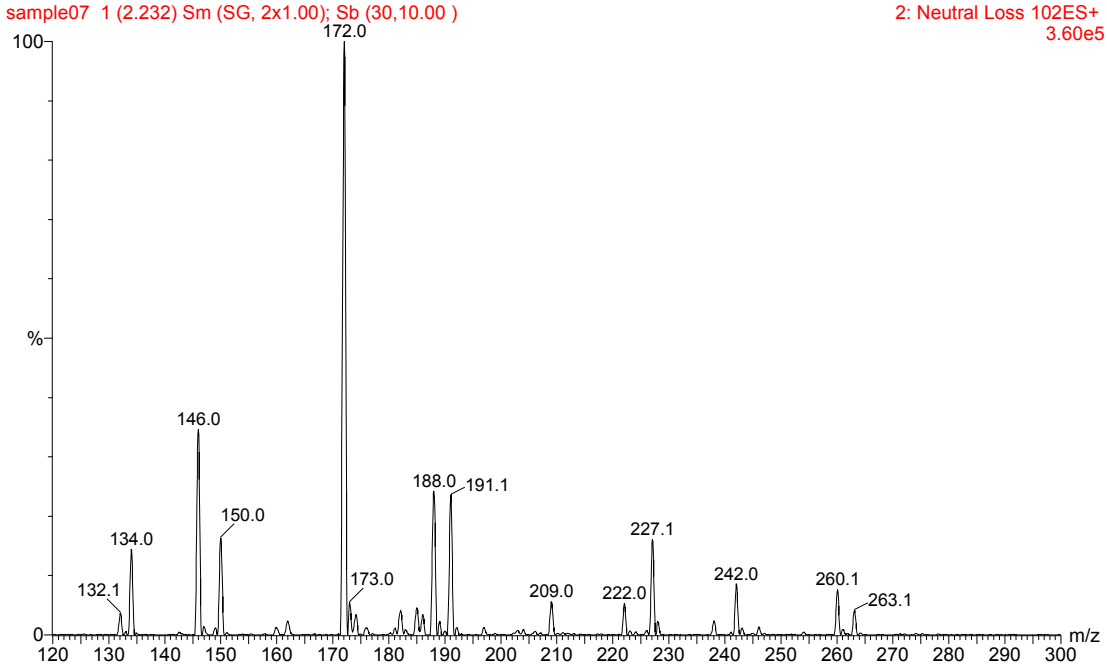
EXAMPLE 3: Spectra for Sample 06



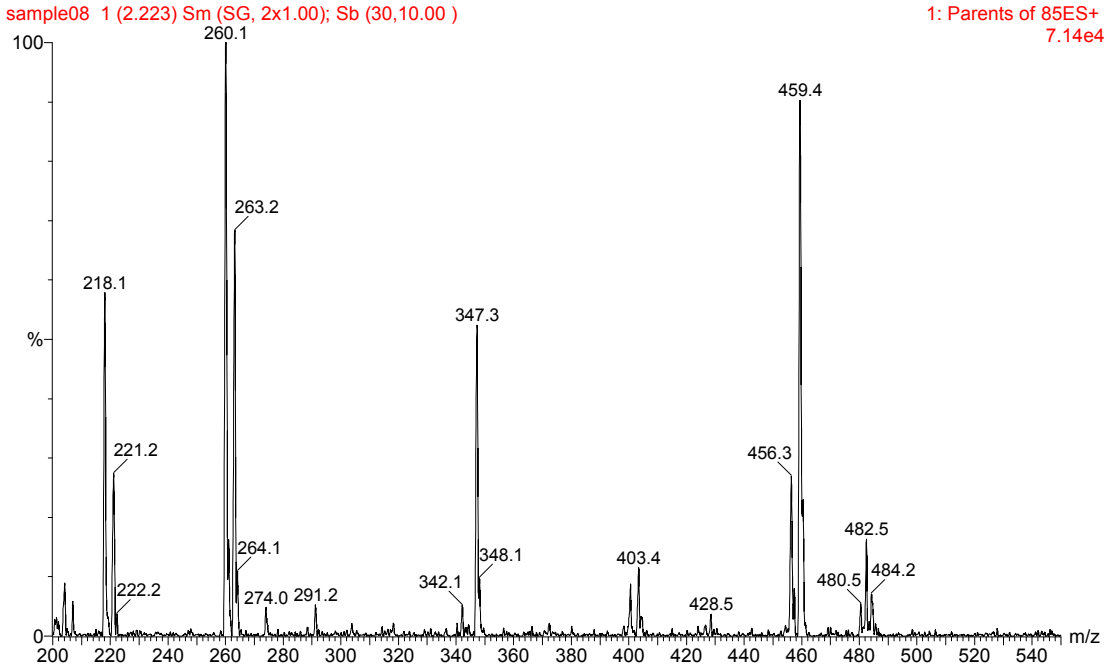


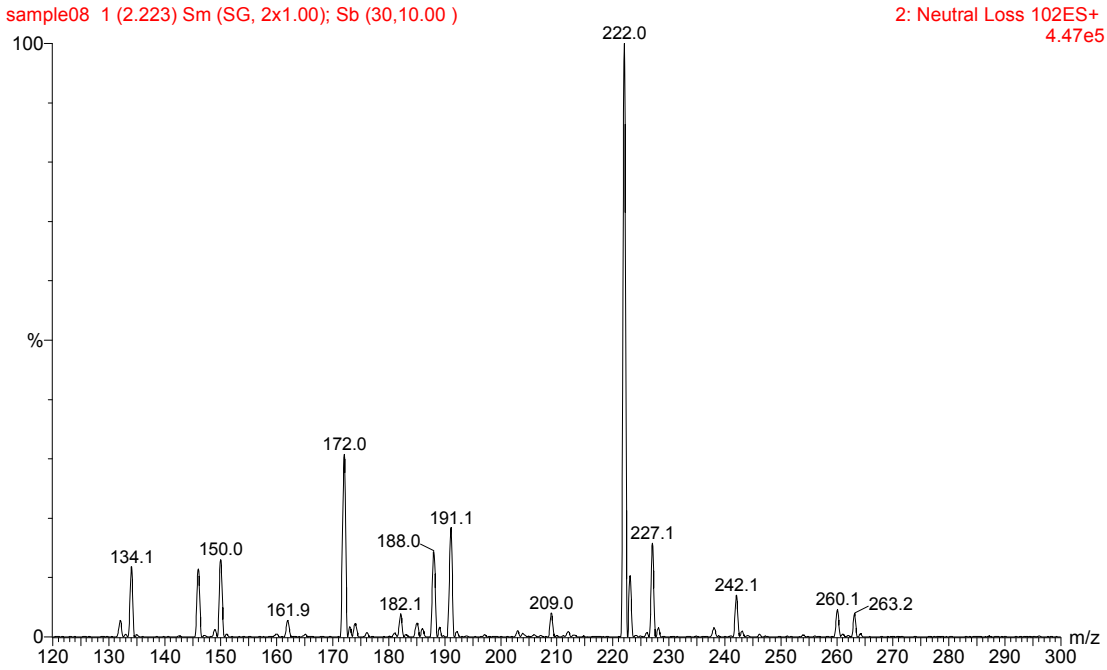
EXAMPLE 3: Spectra for Sample 07





EXAMPLE 3: Spectra for Sample 08





EXAMPLE 4: PROCESS SAMPLES ACQUIRED IN A 2-FUNCTION MRM EXPERIMENT

Objective: To input the appropriate parameters to automatically process data acquired in Multiple Reaction Monitoring mode using the NeoLynx processing.

A series of test samples were prepared using blood samples that had been spiked with analytes of interest, and appropriate amounts of internal standards. The samples were first analyzed in MCA mode (MCA TEST 01 - MCA TEST 11), diluted with mobile phase and re-analysed using MRM mode (MRM TEST 01 - MRM TEST 11).

The following internal standard compounds were used for these particular analyses. In this instance, the levels of the internal standards added are quoted nominally at 10uM/L as the purpose of the exercise is to notice trends in the data files.

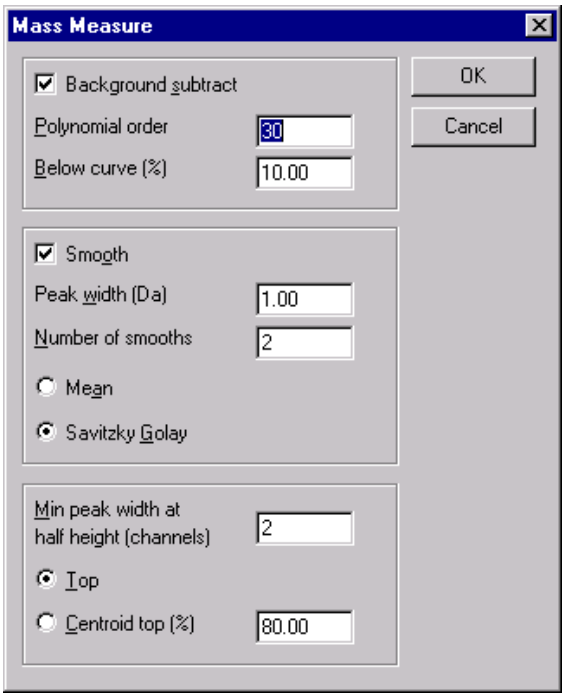
d9-Free carnitine	10μM/L
d3-C2-carnitine	10uM/L
d3-C3-carnitine	10uM/L
d3-C4-carnitine	10uM/L
d3-C8-carnitine	10uM/L
d3-C16-carnitine	10uM/L
d8-valine	10uM/L
d5-phenylalanine	10uM/L
d3-leucine	10uM/L
d3-methionine	10uM/L
d4-tyrosine	10uM/L

- Examine the MRM chromatograms manually, and determine appropriate spectrum combine and background subtract parameters.
- Examine a 'typical' reconstructed spectrum and determine the peak intensity ratios that should be calculated.
- Automatically process the MRM data files and examine the Concentration Summary reports to look for trends.
- Record the trends.
- Using the same set of rules, automatically process all the MCA and MRM files in a single Sample List.
- Check that the same trends are observed for each (MRM *vs* MCA) data set.

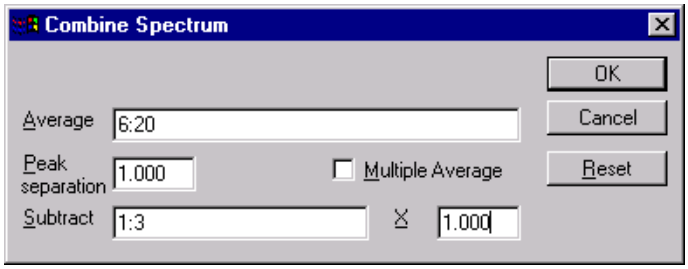
WORKINGS TO EXAMPLES

The workings provided here are an example of one way of processing the data, and are used for illustration only. The tables defined below were those used to achieve the results given and agree with the manual interpretation.

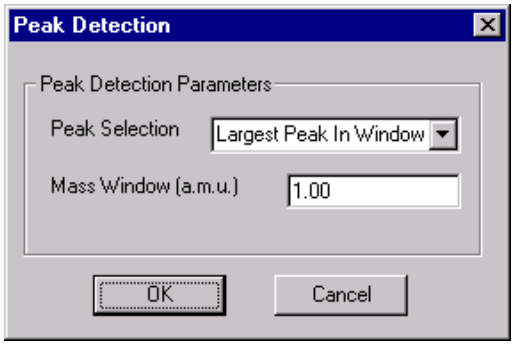
For MCA data (Examples 1 to 3), the Mass Measure parameters used were:



For the MRM data (Example 4), the Spectrum Combine parameters used were:

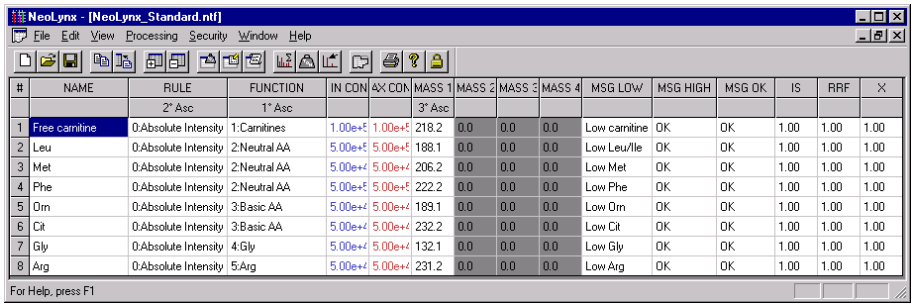


For all the processing, the Peak Detection parameters:



WORKED EXAMPLE 1:

The following rule table (NeoLynx_Standard.ntf) was used:



#	NAME	RULE	FUNCTION	IN CON	AX CON	MASS 1	MASS 2	MASS 3	MASS 4	MSG LOW	MSG HIGH	MSG OK	IS	RRF	X
		2° Asc	1° Asc			3° Asc									
1	Free carnitine	0:Absolute Intensity	1:Carnitines	1.00e+5	1.00e+5	218.2	0.0	0.0	0.0	Low carnitine	OK	OK	1.00	1.00	1.00
2	Leu	0:Absolute Intensity	2:Neutral AA	5.00e+5	5.00e+5	188.1	0.0	0.0	0.0	Low Leu/Ile	OK	OK	1.00	1.00	1.00
3	Met	0:Absolute Intensity	2:Neutral AA	5.00e+4	5.00e+4	206.2	0.0	0.0	0.0	Low Met	OK	OK	1.00	1.00	1.00
4	Phe	0:Absolute Intensity	2:Neutral AA	5.00e+5	5.00e+5	222.2	0.0	0.0	0.0	Low Phe	OK	OK	1.00	1.00	1.00
5	Orn	0:Absolute Intensity	3:Basic AA	5.00e+4	5.00e+4	189.1	0.0	0.0	0.0	Low Orn	OK	OK	1.00	1.00	1.00
6	Cit	0:Absolute Intensity	3:Basic AA	5.00e+4	5.00e+4	232.2	0.0	0.0	0.0	Low Cit	OK	OK	1.00	1.00	1.00
7	Gly	0:Absolute Intensity	4:Gly	5.00e+4	5.00e+4	132.1	0.0	0.0	0.0	Low Gly	OK	OK	1.00	1.00	1.00
8	Arg	0:Absolute Intensity	5:Arg	5.00e+4	5.00e+4	231.2	0.0	0.0	0.0	Low Arg	OK	OK	1.00	1.00	1.00

For Help, press F1

The peak intensities are examined in each mass spectral function, and messages are yielded if the peak intensity for a particular compound is below than the minimum specified threshold (see column).

Note that a high Maximum Threshold value must be set for the rules to work.

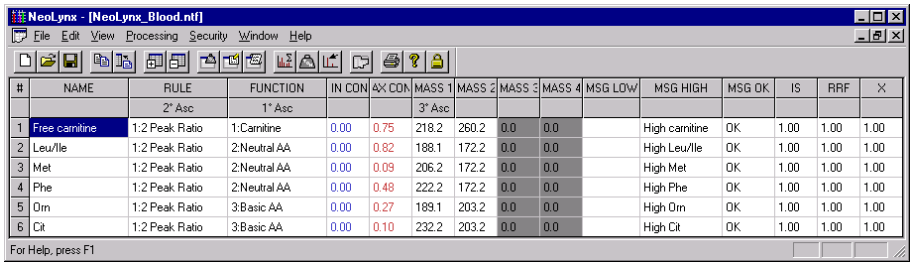
The following shows the sample compositions:

Standard01	Carnitine, Phe, Cit, Orn, Gly, Arg	Missing: (Leu, Met)
Standard02	Carnitine, Phe, Cit, Orn, Gly, Arg	Missing: (Met, Orn)
Standard03	Carnitine, Phe, Orn, Arg	Missing: (Leu, Met, Cit, Gly)
Standard04	Phe, Leu, Cit, Arg	Missing: (Carnitine, Met, Orn, Gly)
Standard05	Carnitine, Phe, Leu, Orn, Gly	Missing: (Met, Cit, Arg)
Standard06	Carnitine, Leu, Met, Cit, Gly	Missing: (Phe, Orn, Arg)
Standard07	Carnitine, Phe, Met, Orn, Arg	Missing: (Leu, Cit, Gly)
Standard08	Phe, Leu, Met, Cit, Arg	Missing: (Carnitine, Orn, Gly)
Standard09	Phe, Met, Orn, Gly, Arg	Missing: (Carnitine, Leu, Cit)
Standard10	Carnitine, Phe, Leu, Met, Orn, Cit, Gly, Arg	

Using the rules, the summary report should show the other compounds (bracketed) as being absent.

WORKED EXAMPLE 2:

The following table (NeoLynx_Blood.ntf) was used. Note that the minimum threshold is not used, as we are only checking for high peak intensity ratios.



#	NAME	RULE	FUNCTION	IN CON	4X CON	MASS 1	MASS 2	MASS 3	MASS 4	MSG LOW	MSG HIGH	MSG OK	IS	RRF	X
		2° Asc	1° Asc			3° Asc									
1	Free carnitine	1:2 Peak Ratio	1:Carnitine	0.00	0.75	218.2	260.2	0.0	0.0		High carnitine	OK	1.00	1.00	1.00
2	Leu/Ile	1:2 Peak Ratio	2:Neutral AA	0.00	0.82	188.1	172.2	0.0	0.0		High Leu/Ile	OK	1.00	1.00	1.00
3	Met	1:2 Peak Ratio	2:Neutral AA	0.00	0.09	206.2	172.2	0.0	0.0		High Met	OK	1.00	1.00	1.00
4	Phe	1:2 Peak Ratio	2:Neutral AA	0.00	0.48	222.2	172.2	0.0	0.0		High Phe	OK	1.00	1.00	1.00
5	Orn	1:2 Peak Ratio	3:Basic AA	0.00	0.27	189.1	203.2	0.0	0.0		High Orn	OK	1.00	1.00	1.00
6	Cit	1:2 Peak Ratio	3:Basic AA	0.00	0.10	232.2	203.2	0.0	0.0		High Cit	OK	1.00	1.00	1.00

The blood samples were doctored in the following way:

Blood01 Normal

Blood02 Normal

Blood03 Added 5uL Phe solution

Blood04 Added 10uL Cit solution

Blood05 Added 10uL Leu solution

Blood06 Normal

Blood07 Added 10uL Met solution

Blood08 Normal

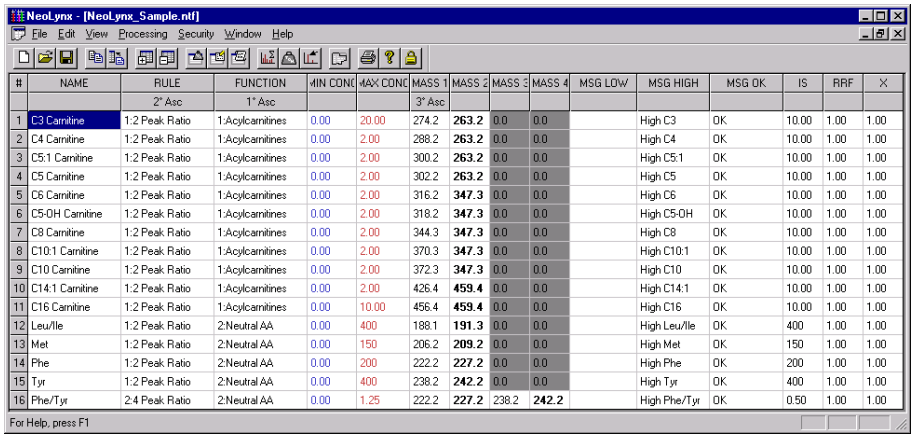
Blood09 Added 5uL of Phe, Leu and Cit solutions

Blood10 Added 20uL Phe solution

Note that comparing the results from Blood03 and Blood10, the reported Phe concentration is approximately 4 times higher for Blood10, as would be expected from the addition.

WORKED EXAMPLE 3:

The following table (NeoLynx_Sample.ntf) was used.



NeoLynx - [NeoLynx_Sample.ntf]

File Edit View Processing Security Window Help

#	NAME	RULE	FUNCTION	MIN CONC	MAX CONC	MASS 1	MASS 2	MASS 3	MASS 4	MSG LOW	MSG HIGH	MSG OK	IS	RRF	X
		2° Asc	1° Asc			3° Asc									
1	C3 Carnitine	1:2 Peak Ratio	1:Acylcarnitines	0.00	20.00	274.2	**263.2**	0.0	0.0		High C3	OK	10.00	1.00	1.00
2	C4 Carnitine	1:2 Peak Ratio	1:Acylcarnitines	0.00	2.00	288.2	**263.2**	0.0	0.0		High C4	OK	10.00	1.00	1.00
3	C5:1 Carnitine	1:2 Peak Ratio	1:Acylcarnitines	0.00	2.00	300.2	**263.2**	0.0	0.0		High C5:1	OK	10.00	1.00	1.00
4	C5 Carnitine	1:2 Peak Ratio	1:Acylcarnitines	0.00	2.00	302.2	**263.2**	0.0	0.0		High C5	OK	10.00	1.00	1.00
5	C6 Carnitine	1:2 Peak Ratio	1:Acylcarnitines	0.00	2.00	316.2	**347.3**	0.0	0.0		High C6	OK	10.00	1.00	1.00
6	C5-OH Carnitine	1:2 Peak Ratio	1:Acylcarnitines	0.00	2.00	318.2	**347.3**	0.0	0.0		High C5-OH	OK	10.00	1.00	1.00
7	C8 Carnitine	1:2 Peak Ratio	1:Acylcarnitines	0.00	2.00	344.3	**347.3**	0.0	0.0		High C8	OK	10.00	1.00	1.00
8	C10:1 Carnitine	1:2 Peak Ratio	1:Acylcarnitines	0.00	2.00	370.3	**347.3**	0.0	0.0		High C10:1	OK	10.00	1.00	1.00
9	C10 Carnitine	1:2 Peak Ratio	1:Acylcarnitines	0.00	2.00	372.3	**347.3**	0.0	0.0		High C10	OK	10.00	1.00	1.00
10	C14:1 Carnitine	1:2 Peak Ratio	1:Acylcarnitines	0.00	2.00	426.4	**459.4**	0.0	0.0		High C14:1	OK	10.00	1.00	1.00
11	C16 Carnitine	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	456.4	**459.4**	0.0	0.0		High C16	OK	10.00	1.00	1.00
12	Leu/Ile	1:2 Peak Ratio	2:Neutral AA	0.00	400	188.1	**191.3**	0.0	0.0		High Leu/Ile	OK	400	1.00	1.00
13	Met	1:2 Peak Ratio	2:Neutral AA	0.00	150	206.2	**209.2**	0.0	0.0		High Met	OK	150	1.00	1.00
14	Phe	1:2 Peak Ratio	2:Neutral AA	0.00	200	222.2	**227.2**	0.0	0.0		High Phe	OK	200	1.00	1.00
15	Tyr	1:2 Peak Ratio	2:Neutral AA	0.00	400	238.2	**242.2**	0.0	0.0		High Tyr	OK	400	1.00	1.00
16	Phe/Tyr	2:4 Peak Ratio	2:Neutral AA	0.00	1.25	222.2	**227.2**	238.2	**242.2**		High Phe/Tyr	OK	0.50	1.00	1.00

For Help, press F1

Note that there is no Minimum Threshold used in this analysis, hence no Message Low.

The following inferences may be drawn:

SAMPLE01 Normal

SAMPLE02 High Leu/Ile, 1064uM/L; MSUD patient

SAMPLE03 High Phe, 457uM/L; probable HyperPhe patient

SAMPLE04 C4, C6, C8, C10, C10:1 carnitines high; MCAD

SAMPLE05 High C3 carnitine; possible propionic acidaemia or MMA

SAMPLE06 Normal

SAMPLE07 High C5-OH carnitine; C4 carnitine not found (OK?); C16 carnitine high?

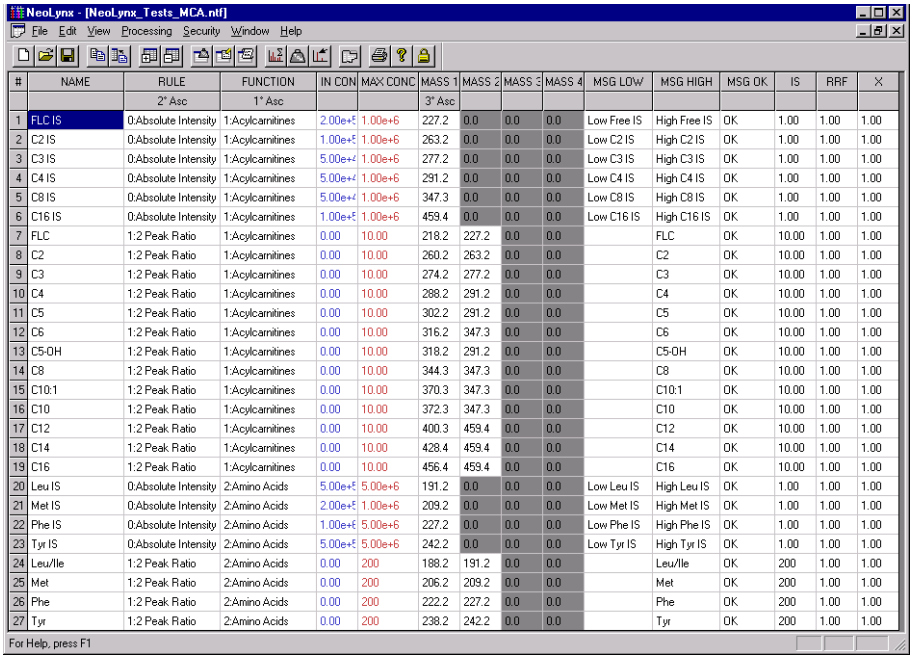
SAMPLE08 High Phe, 1264uM/L. [Phe]/[Tyr] also high, 14.5. Note use of IS ratio.

The summary report for the Sample01-Sample08 files in the NeoLynx generic formats are shown below in an Excel interpretation.

WORKED EXAMPLE 4

The most important thing to note in this example is that a peak appearing in the MRM spectrum will appear in the MCA trace, but a peak appearing in an MCA trace will only appear in the MRM result if it has been programmed.

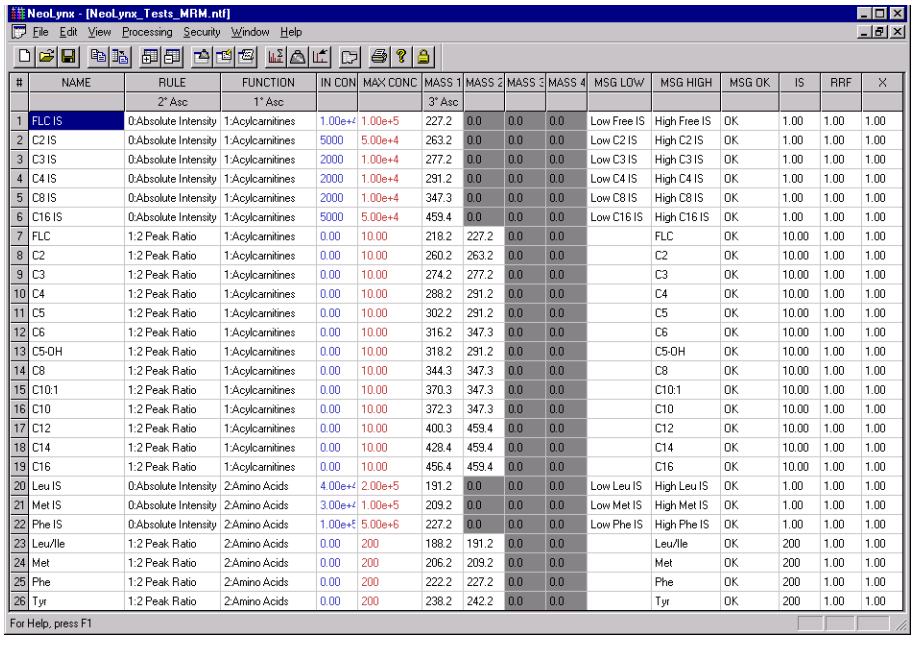
The following rule tables, NeoLynx_Test_MRM.ntf and NeoLynx_Test_MCA.ntf, were used (different signal intensity thresholds from the MRM and MCA experiments require different NeoLynx test files to be used in a single sample list):


NeoLynx - [NeoLynx_Tests_MCA.ntf]

File Edit View Processing Security Window Help

#	NAME	RULE	FUNCTION	IN CON	MAX CONC	MASS 1	MASS 2	MASS 3	MASS 4	MSG LOW	MSG HIGH	MSG OK	IS	RRF	X
		2° Asc	1° Asc			3° Asc									
1	FLC IS	0:Absolute Intensity	1:Acylcarnitines	2.00e+5	1.00e+6	227.2	0.0	0.0	0.0	Low Free IS	High Free IS	OK	1.00	1.00	1.00
2	C2 IS	0:Absolute Intensity	1:Acylcarnitines	1.00e+5	1.00e+6	263.2	0.0	0.0	0.0	Low C2 IS	High C2 IS	OK	1.00	1.00	1.00
3	C3 IS	0:Absolute Intensity	1:Acylcarnitines	5.00e+4	1.00e+6	277.2	0.0	0.0	0.0	Low C3 IS	High C3 IS	OK	1.00	1.00	1.00
4	C4 IS	0:Absolute Intensity	1:Acylcarnitines	5.00e+4	1.00e+6	291.2	0.0	0.0	0.0	Low C4 IS	High C4 IS	OK	1.00	1.00	1.00
5	C8 IS	0:Absolute Intensity	1:Acylcarnitines	5.00e+4	1.00e+6	347.3	0.0	0.0	0.0	Low C8 IS	High C8 IS	OK	1.00	1.00	1.00
6	C16 IS	0:Absolute Intensity	1:Acylcarnitines	1.00e+5	1.00e+6	459.4	0.0	0.0	0.0	Low C16 IS	High C16 IS	OK	1.00	1.00	1.00
7	FLC	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	218.2	227.2	0.0	0.0		FLC	OK	10.00	1.00	1.00
8	C2	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	260.2	263.2	0.0	0.0		C2	OK	10.00	1.00	1.00
9	C3	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	274.2	277.2	0.0	0.0		C3	OK	10.00	1.00	1.00
10	C4	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	288.2	291.2	0.0	0.0		C4	OK	10.00	1.00	1.00
11	C5	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	302.2	291.2	0.0	0.0		C5	OK	10.00	1.00	1.00
12	C6	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	316.2	347.3	0.0	0.0		C6	OK	10.00	1.00	1.00
13	C5-OH	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	318.2	291.2	0.0	0.0		C5-OH	OK	10.00	1.00	1.00
14	C8	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	344.3	347.3	0.0	0.0		C8	OK	10.00	1.00	1.00
15	C10:1	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	370.3	347.3	0.0	0.0		C10:1	OK	10.00	1.00	1.00
16	C10	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	372.3	347.3	0.0	0.0		C10	OK	10.00	1.00	1.00
17	C12	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	400.3	459.4	0.0	0.0		C12	OK	10.00	1.00	1.00
18	C14	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	428.4	459.4	0.0	0.0		C14	OK	10.00	1.00	1.00
19	C16	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	456.4	459.4	0.0	0.0		C16	OK	10.00	1.00	1.00
20	Leu IS	0:Absolute Intensity	2:Amino Acids	5.00e+5	5.00e+6	191.2	0.0	0.0	0.0	Low Leu IS	High Leu IS	OK	1.00	1.00	1.00
21	Met IS	0:Absolute Intensity	2:Amino Acids	2.00e+5	1.00e+6	209.2	0.0	0.0	0.0	Low Met IS	High Met IS	OK	1.00	1.00	1.00
22	Phe IS	0:Absolute Intensity	2:Amino Acids	1.00e+5	5.00e+6	227.2	0.0	0.0	0.0	Low Phe IS	High Phe IS	OK	1.00	1.00	1.00
23	Tyr IS	0:Absolute Intensity	2:Amino Acids	5.00e+5	5.00e+6	242.2	0.0	0.0	0.0	Low Tyr IS	High Tyr IS	OK	1.00	1.00	1.00
24	Leu/Ile	1:2 Peak Ratio	2:Amino Acids	0.00	200	188.2	191.2	0.0	0.0		Leu/Ile	OK	200	1.00	1.00
25	Met	1:2 Peak Ratio	2:Amino Acids	0.00	200	206.2	209.2	0.0	0.0		Met	OK	200	1.00	1.00
26	Phe	1:2 Peak Ratio	2:Amino Acids	0.00	200	222.2	227.2	0.0	0.0		Phe	OK	200	1.00	1.00
27	Tyr	1:2 Peak Ratio	2:Amino Acids	0.00	200	238.2	242.2	0.0	0.0		Tyr	OK	200	1.00	1.00

For Help, press F1


NeoLynx - [NeoLynx_Tests_MRM.ntf]

File Edit View Processing Security Window Help

#	NAME	RULE	FUNCTION	IN CON	MAX CONC	MASS 1	MASS 2	MASS 3	MASS 4	MSG LOW	MSG HIGH	MSG OK	IS	RRF	X
		2° Asc	1° Asc			3° Asc									
1	FLC IS	0:Absolute Intensity	1:Acylcarnitines	1.00e+4	1.00e+5	227.2	0.0	0.0	0.0	Low Free IS	High Free IS	OK	1.00	1.00	1.00
2	C2 IS	0:Absolute Intensity	1:Acylcarnitines	5000	5.00e+4	263.2	0.0	0.0	0.0	Low C2 IS	High C2 IS	OK	1.00	1.00	1.00
3	C3 IS	0:Absolute Intensity	1:Acylcarnitines	2000	1.00e+4	277.2	0.0	0.0	0.0	Low C3 IS	High C3 IS	OK	1.00	1.00	1.00
4	C4 IS	0:Absolute Intensity	1:Acylcarnitines	2000	1.00e+4	291.2	0.0	0.0	0.0	Low C4 IS	High C4 IS	OK	1.00	1.00	1.00
5	C8 IS	0:Absolute Intensity	1:Acylcarnitines	2000	1.00e+4	347.3	0.0	0.0	0.0	Low C8 IS	High C8 IS	OK	1.00	1.00	1.00
6	C16 IS	0:Absolute Intensity	1:Acylcarnitines	5000	5.00e+4	459.4	0.0	0.0	0.0	Low C16 IS	High C16 IS	OK	1.00	1.00	1.00
7	FLC	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	218.2	227.2	0.0	0.0		FLC	OK	10.00	1.00	1.00
8	C2	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	260.2	263.2	0.0	0.0		C2	OK	10.00	1.00	1.00
9	C3	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	274.2	277.2	0.0	0.0		C3	OK	10.00	1.00	1.00
10	C4	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	288.2	291.2	0.0	0.0		C4	OK	10.00	1.00	1.00
11	C5	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	302.2	291.2	0.0	0.0		C5	OK	10.00	1.00	1.00
12	C6	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	316.2	347.3	0.0	0.0		C6	OK	10.00	1.00	1.00
13	C5-OH	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	318.2	291.2	0.0	0.0		C5-OH	OK	10.00	1.00	1.00
14	C8	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	344.3	347.3	0.0	0.0		C8	OK	10.00	1.00	1.00
15	C10:1	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	370.3	347.3	0.0	0.0		C10:1	OK	10.00	1.00	1.00
16	C10	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	372.3	347.3	0.0	0.0		C10	OK	10.00	1.00	1.00
17	C12	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	400.3	459.4	0.0	0.0		C12	OK	10.00	1.00	1.00
18	C14	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	428.4	459.4	0.0	0.0		C14	OK	10.00	1.00	1.00
19	C16	1:2 Peak Ratio	1:Acylcarnitines	0.00	10.00	456.4	459.4	0.0	0.0		C16	OK	10.00	1.00	1.00
20	Leu IS	0:Absolute Intensity	2:Amino Acids	4.00e+4	2.00e+5	191.2	0.0	0.0	0.0	Low Leu IS	High Leu IS	OK	1.00	1.00	1.00
21	Met IS	0:Absolute Intensity	2:Amino Acids	3.00e+4	1.00e+5	209.2	0.0	0.0	0.0	Low Met IS	High Met IS	OK	1.00	1.00	1.00
22	Phe IS	0:Absolute Intensity	2:Amino Acids	1.00e+5	5.00e+6	227.2	0.0	0.0	0.0	Low Phe IS	High Phe IS	OK	1.00	1.00	1.00
23	Leu/Ile	1:2 Peak Ratio	2:Amino Acids	0.00	200	188.2	191.2	0.0	0.0		Leu/Ile	OK	200	1.00	1.00
24	Met	1:2 Peak Ratio	2:Amino Acids	0.00	200	206.2	209.2	0.0	0.0		Met	OK	200	1.00	1.00
25	Phe	1:2 Peak Ratio	2:Amino Acids	0.00	200	222.2	227.2	0.0	0.0		Phe	OK	200	1.00	1.00
26	Tyr	1:2 Peak Ratio	2:Amino Acids	0.00	200	238.2	242.2	0.0	0.0		Tyr	OK	200	1.00	1.00

For Help, press F1

Note that only a nominal Maximum Threshold has been used in this example as the results should be viewed from the summary file generated - see below for the report styles. If the results are examined using the CONC1.rep style, the following trends should be noted:

TEST 01	Blank sample - should be returned as the internal standard levels being too low. This rule has not been included in the above table, but should really be considered to differentiate real samples from blanks - see Example 1.
TEST 02	Endogenous blood sample
TEST 03-06	Increasing levels of Phe and Tyr should be observed, with the additions being in the proportion, 2, 4, 8, 12. Met, Leu/Ile and Val levels should remain reasonably constant.
TEST 07-11	Increasing levels of some acylcarnitines should be observed. Additions have been made for C2, C3, C5, C6, C8, C10, C12, C14, C16 and C18. A general trend only should be recorded as increasing with increasing file name number.

Appendix D - Importing Worksheets

Overview

Information can be imported into a MassLynx Sample List in a number of ways. The Sample Lists chapter in the MassLynx NT User's Guide contains a full explanation of all methods. This chapter describes the simplest way – defining data in a spreadsheet, saving it as a tab-delimited text file and importing the text file.

Text file data can be imported in one of the following ways.

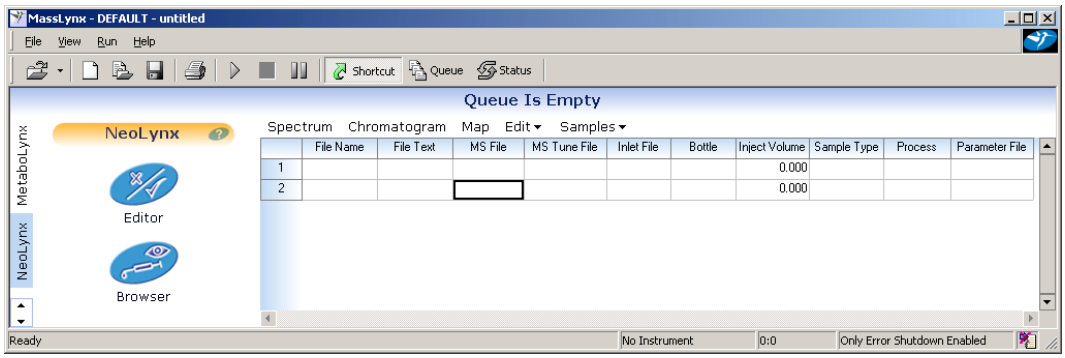
Import Worksheet This requires an index column and a specific format for the first row in the imported file.

Import Data This does not require an index field or a formatted first row but does need to be imported into a formatted sample list

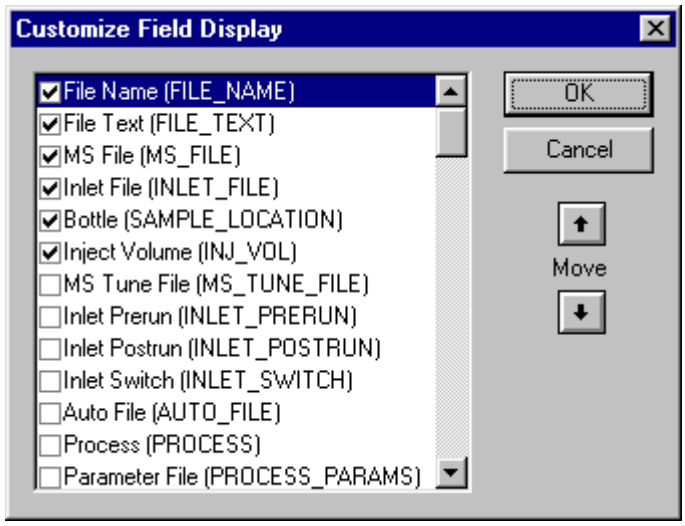
Import Worksheet

Load the required sample list format (**Samples, Load Format**). This defines the appropriate columns for the analyses to be performed.

Select **File, New**.

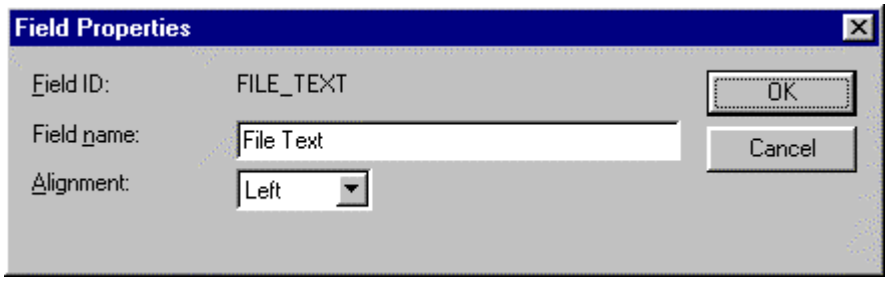


The Field ID of the columns must be used on the first line of the file created. To determine the Field IDs select Samples, Format, Customize, from Sample List Menu Bar, to invoke the Customize Field Display dialog, alternatively right click on the sample list and select Customize Display from the pop up menu.



The name in brackets is the Field ID.

Another way of determining the Field ID is to click with the right mouse button on column header and select Properties from the pop up menu displayed. The Field Properties dialog is displayed with the Field ID shown on the top line. In the example shown the Field ID is 'FILE_TEXT'.



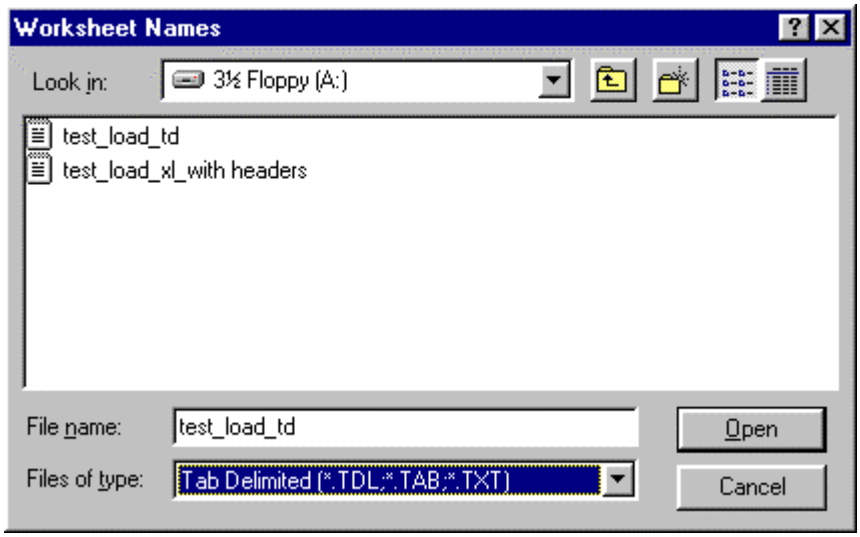
Prepare the data in an external spreadsheet package as shown below.

Note: the first cell should contain the text 'INDEX' the following cells in the row should contain the Field ID text.

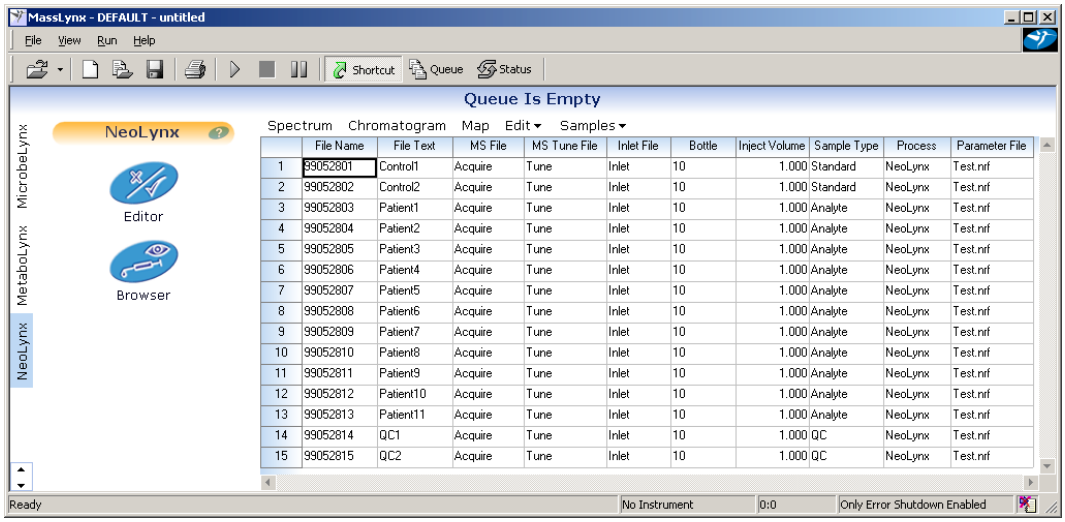
	A	B	C	D	E	F	G	H	I	J	K
1	INDEX	FILE_NAME	FILE_TEXT	MS_FILE	MS_TUNE_FILE	INLET_FILE	INJ_VOL	SAMPLE_LOCATION	TYPE	PROCESS	PROCESS_PARAMS
2	1	99052801	Control 1	Acquire	Tune	Inlet	10	1,1:A,1	Standard	NeoLynx	Test.ntf
3	2	99052802	Control 2	Acquire	Tune	Inlet	10	1,1:A,2	Standard	NeoLynx	Test.ntf
4	3	99052803	Patient 1	Acquire	Tune	Inlet	10	1,1:A,3	Analyte	NeoLynx	Test.ntf
5	4	99052804	Patient 2	Acquire	Tune	Inlet	10	1,1:A,4	Analyte	NeoLynx	Test.ntf
6	5	99052805	Patient 3	Acquire	Tune	Inlet	10	1,1:A,5	Analyte	NeoLynx	Test.ntf
7	6	99052806	Patient 4	Acquire	Tune	Inlet	10	1,1:A,6	Analyte	NeoLynx	Test.ntf
8	7	99052807	Patient 5	Acquire	Tune	Inlet	10	1,1:A,7	Analyte	NeoLynx	Test.ntf
9	8	99052808	Patient 6	Acquire	Tune	Inlet	10	1,1:A,8	Analyte	NeoLynx	Test.ntf
10	9	99052809	Patient 7	Acquire	Tune	Inlet	10	1,1:A,9	Analyte	NeoLynx	Test.ntf
11	10	99052810	Patient 18	Acquire	Tune	Inlet	10	1,1:A,10	Analyte	NeoLynx	Test.ntf
12	11	99052811	Patient 19	Acquire	Tune	Inlet	10	1,1:A,11	Analyte	NeoLynx	Test.ntf
13	12	99052812	Patient 10	Acquire	Tune	Inlet	10	1,1:A,12	Analyte	NeoLynx	Test.ntf
14	13	99052813	Patient 11	Acquire	Tune	Inlet	10	1,1:B,1	Analyte	NeoLynx	Test.ntf
15	14	99052814	QC 1	Acquire	Tune	Inlet	10	1,1:B,2	QC	NeoLynx	Test.ntf
16	15	99052815	QC 2	Acquire	Tune	Inlet	10	1,1:B,3	QC	NeoLynx	Test.ntf
17											

When a file has been prepared (e.g. in Excel) save it as a tab-delimited text file (*.txt). This file may then be directly imported as a Worksheet without modification.

From the MassLynx top level, select File, Import Worksheet and select Tab Delimited from the Files of type box.



Select the file to be imported and press Open. The example file shown above will be imported into the sample list as.



This Sample List may then be saved and run in the normal way.

Import Data

This method works in a similar way to that described above.

Load the required sample list format (Samples, Load Format). This defines the appropriate columns for the analyses to be performed.

Select File, New.

Prepare the data in an external spreadsheet package as shown below.

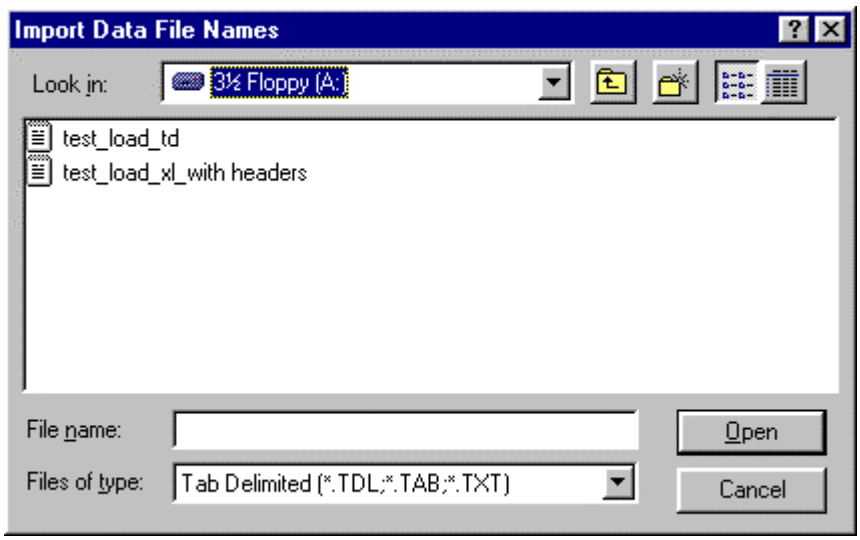
	A	B	C	D	E	F	G	H	I	J
1	99052801	Control 1	Acquire	Tune	Inlet	10	1,1:A,1	Standard	NeoLynx	Test.ntf
2	99052802	Control 2	Acquire	Tune	Inlet	10	1,1:A,2	Standard	NeoLynx	Test.ntf
3	99052803	Patient 1	Acquire	Tune	Inlet	10	1,1:A,3	Analyte	NeoLynx	Test.ntf
4	99052804	Patient 2	Acquire	Tune	Inlet	10	1,1:A,4	Analyte	NeoLynx	Test.ntf
5	99052805	Patient 3	Acquire	Tune	Inlet	10	1,1:A,5	Analyte	NeoLynx	Test.ntf
6	99052806	Patient 4	Acquire	Tune	Inlet	10	1,1:A,6	Analyte	NeoLynx	Test.ntf
7	99052807	Patient 5	Acquire	Tune	Inlet	10	1,1:A,7	Analyte	NeoLynx	Test.ntf
8	99052808	Patient 6	Acquire	Tune	Inlet	10	1,1:A,8	Analyte	NeoLynx	Test.ntf
9	99052809	Patient 7	Acquire	Tune	Inlet	10	1,1:A,9	Analyte	NeoLynx	Test.ntf
10	99052810	Patient 18	Acquire	Tune	Inlet	10	1,1:A,10	Analyte	NeoLynx	Test.ntf
11	99052811	Patient 19	Acquire	Tune	Inlet	10	1,1:A,11	Analyte	NeoLynx	Test.ntf
12	99052812	Patient 10	Acquire	Tune	Inlet	10	1,1:A,12	Analyte	NeoLynx	Test.ntf
13	99052813	Patient 11	Acquire	Tune	Inlet	10	1,1:B,1	Analyte	NeoLynx	Test.ntf
14										

Note: The order of the data in spreadsheet must match the order of the columns in the sample list.

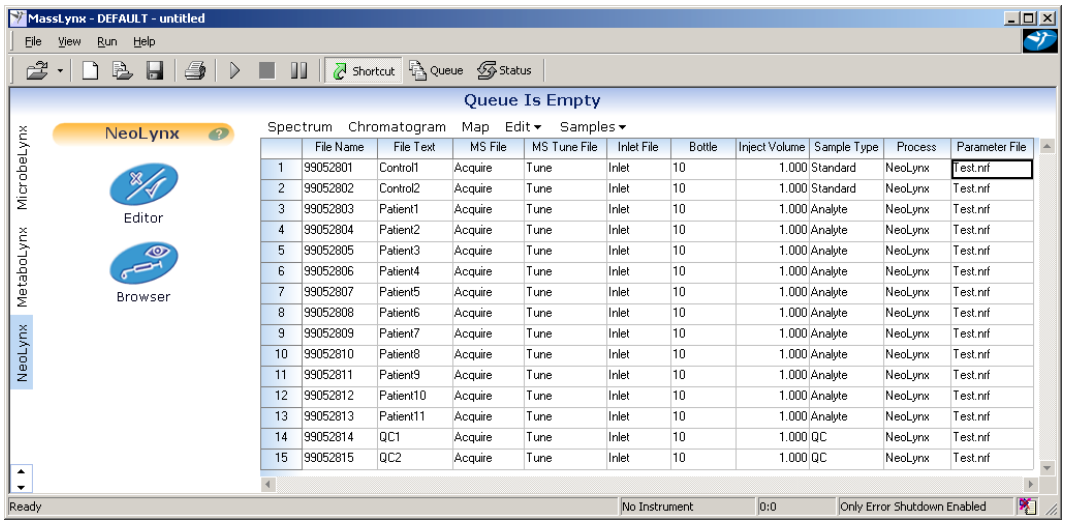
Count the number of row containing data (13 in this case) and ensure that the Sample List has the same number. If the sample list is prepared with too few rows, some sample data will not be imported and the samples will not analyzed. If a sample list is prepared with too many rows some rows will be blank and the sample list will not run.

When a file has been prepared (e.g. in Excel) save it as a tab-delimited text file (*.txt).

From the MassLynx top level, select File, Import Worksheet and select Tab Delimited from the Files of type box.



Select the file to be imported and press Open. The example file shown above will be imported into the sample list as.



The Sample List may now be saved and run in the normal way.

Appendix E NRS Configurations

ELECTRONIC SUMMARY REPORT INFORMATION

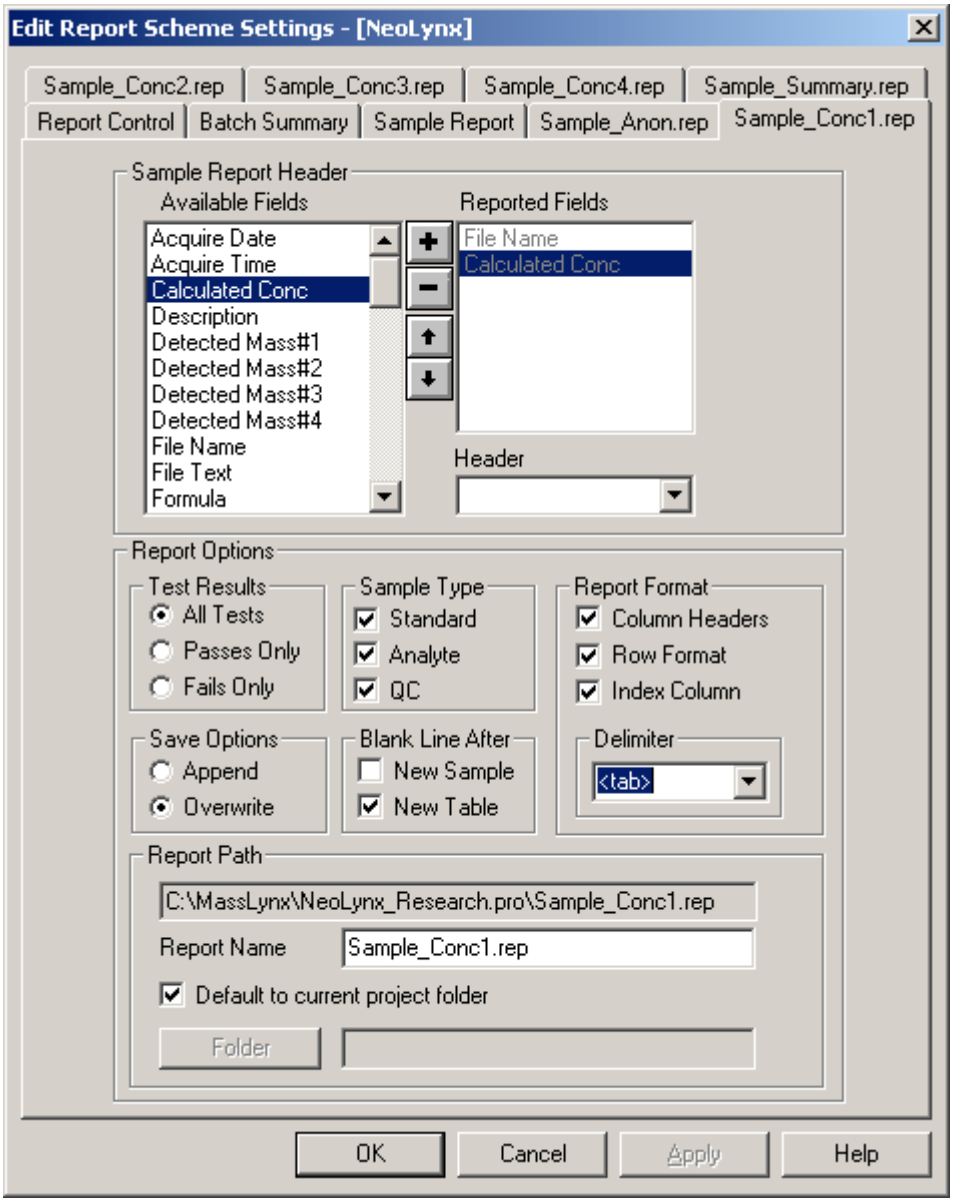
This section shows the layout of the six Electronic Reports generated using the NeoLynx Results File (Sample_001.nrf) and the NeoLynx Results Scheme (NeoLynx.nrs). It is intended to act as a starting point for defining reports and as a reference to the default settings for restoring the original report in case inappropriate values have been entered.

The NeoLynx.nrs scheme is supplied with the NeoLynx software and is designed to produce reports of a similar format to previous versions of NeoLynx.

For all reports the sample list name can be included in the Report Name by entering %Sample% in the Report Name field. This will make reports unique to a sample list. E.g. if the sample list name is Blood.spl then the reports will be Blood_Conc1.rep, Blood_Conc2.rep etc.

To make the result summaries below fit easily on the page, the word 'Carnitine' has been omitted from the appropriate cells.

Sample_Conc1.rep



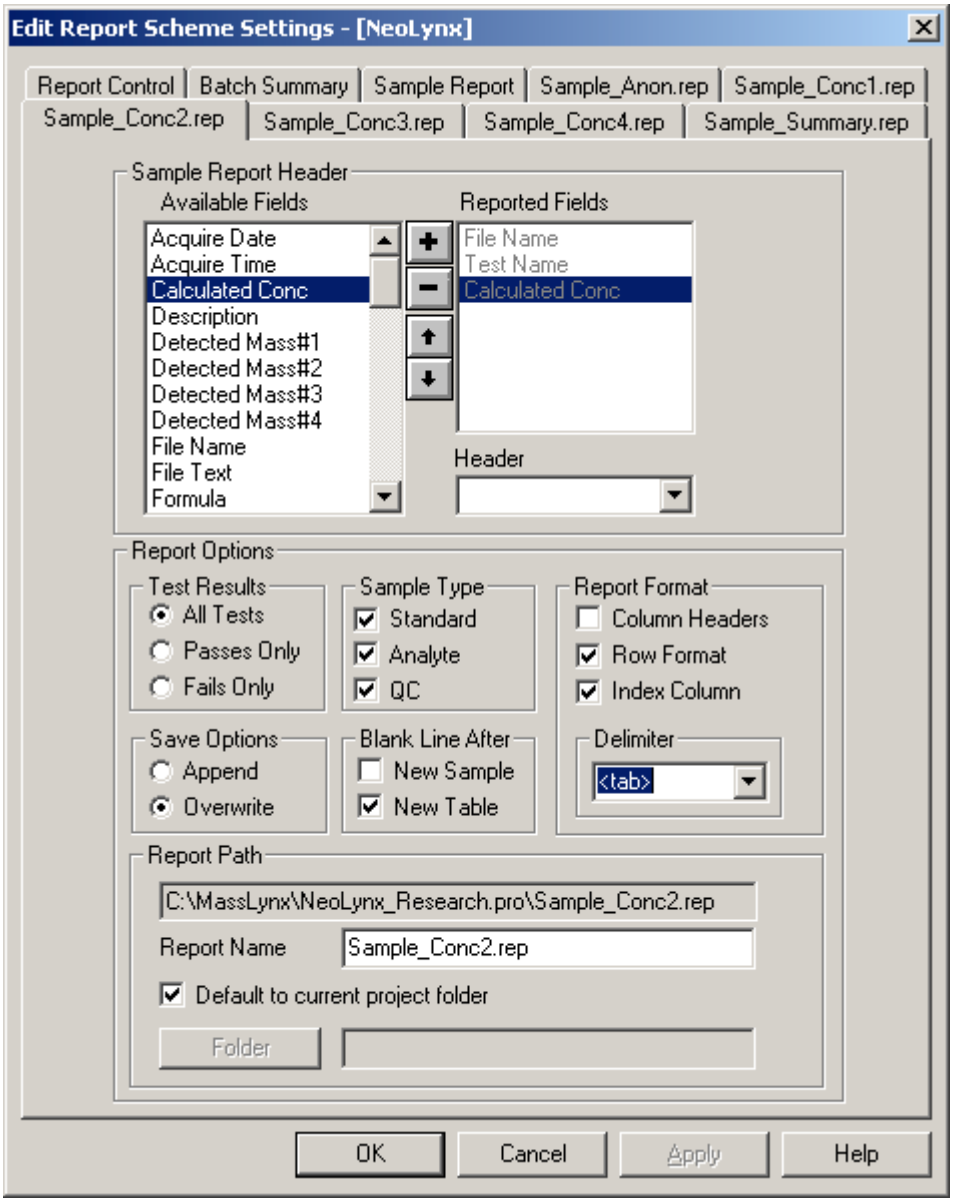
The left column contains the filename from the sample list. The top row contains name of test, and the body of the table contains the calculated analyte concentrations. The report has been split into two sections for ease of viewing, with the filename column being repeated for each section. The file is tab-delimited.

If viewed in a spreadsheet package accepting tab-delimited files, Sample_Conc1.rep will have the appearance:

	C3 Carnitine	C4 Carnitine	C5:1 Carnitine	C5 Carnitine	C6 Carnitine	C5-OH Carnitine	C8 Carnitine	C10:1 Carnitine	C10 Carnitine
Sample01	1.51	0.12	0.04	0.06	0.3	0.35	0.53	0.68	0.37
Sample02	0.89	0.17	0.24	0.11	0.17	0.31	0.53	0.46	0.6
Sample03	2.07	0.39	0.25	0.29	0.23	0.44	0.3	0.42	0.38
Sample04	1.68	2.73	0.08	0.24	6	1.18	34.51	3.39	4.18
Sample05	23.55	0.34	0.27	0.33	0.23	0.38	0.31	0.15	0.37
Sample06	2.25	0.41	0.09	0.49	0.21	1.07	0.97	0.81	0.68
Sample07	1.63	0	0.34	0.5	0.46	11.83	1.68	1.46	1.67
Sample08	0.7	0.22	0.08	0.14	0.21	0.41	0.38	0.17	0.41
Sample101	1.51	0.12	0.04	0.06	0.3	0.35	0.53	0.68	0.37
Sample102	0.89	0.17	0.24	0.11	0.17	0.31	0.53	0.46	0.6
Sample103	2.07	0.39	0.25	0.29	0.23	0.44	0.3	0.42	0.38
Sample104	1.68	2.73	0.08	0.24	6	1.18	34.51	3.39	4.18
Sample105	23.55	0.34	0.27	0.33	0.23	0.38	0.31	0.15	0.37
Sample106	2.25	0.41	0.09	0.49	0.21	1.07	0.97	0.81	0.68
Sample107	1.63	0	0.34	0.5	0.46	11.83	1.68	1.46	1.67
Sample108	0.7	0.22	0.08	0.14	0.21	0.41	0.38	0.17	0.41

	C14:1 Carnitine	C16 Carnitine	Leu/Ile	Met	Phe	Tyr	Phe/Tyr
Sample01	0.17	1.42	155.06	12.03	41.7	127.64	0.33
Sample02	0.25	0.93	1064.37	6.34	46.1	81.75	0.56
Sample03	0.22	3.36	181.58	10.45	457.43	103.22	4.43
Sample04	0.23	3.26	174.43	14.33	61.78	176.63	0.35
Sample05	0.59	1.57	304.99	15.78	35.96	56.81	0.63
Sample06	1.81	8.03	183.48	12.99	37.3	168.52	0.22
Sample07	0.89	10.09	412.48	15.63	65.74	109.45	0.6
Sample08	0.2	2.98	312.09	13.83	1264.24	87.25	14.49
Sample101	0.17	1.42	155.06	12.03	41.7	127.64	0.33
Sample102	0.25	0.93	1064.37	6.34	46.1	81.75	0.56
Sample103	0.22	3.36	181.58	10.45	457.43	103.22	4.43
Sample104	0.23	3.26	174.43	14.33	61.78	176.63	0.35
Sample105	0.59	1.57	304.99	15.78	35.96	56.81	0.63
Sample106	1.81	8.03	183.48	12.99	37.3	168.52	0.22
Sample107	0.89	10.09	412.48	15.63	65.74	109.45	0.6
Sample108	0.2	2.98	312.09	13.83	1264.24	87.25	14.49

Sample_Conc2.rep



The information in Samples_Conc1.rep is reformatted to show one sample per line. The line contains the file name followed by each test name and the associated analyte concentration. This report has been split into three sections for ease of viewing, with the filename being repeated for each section. The file is tab-delimited.

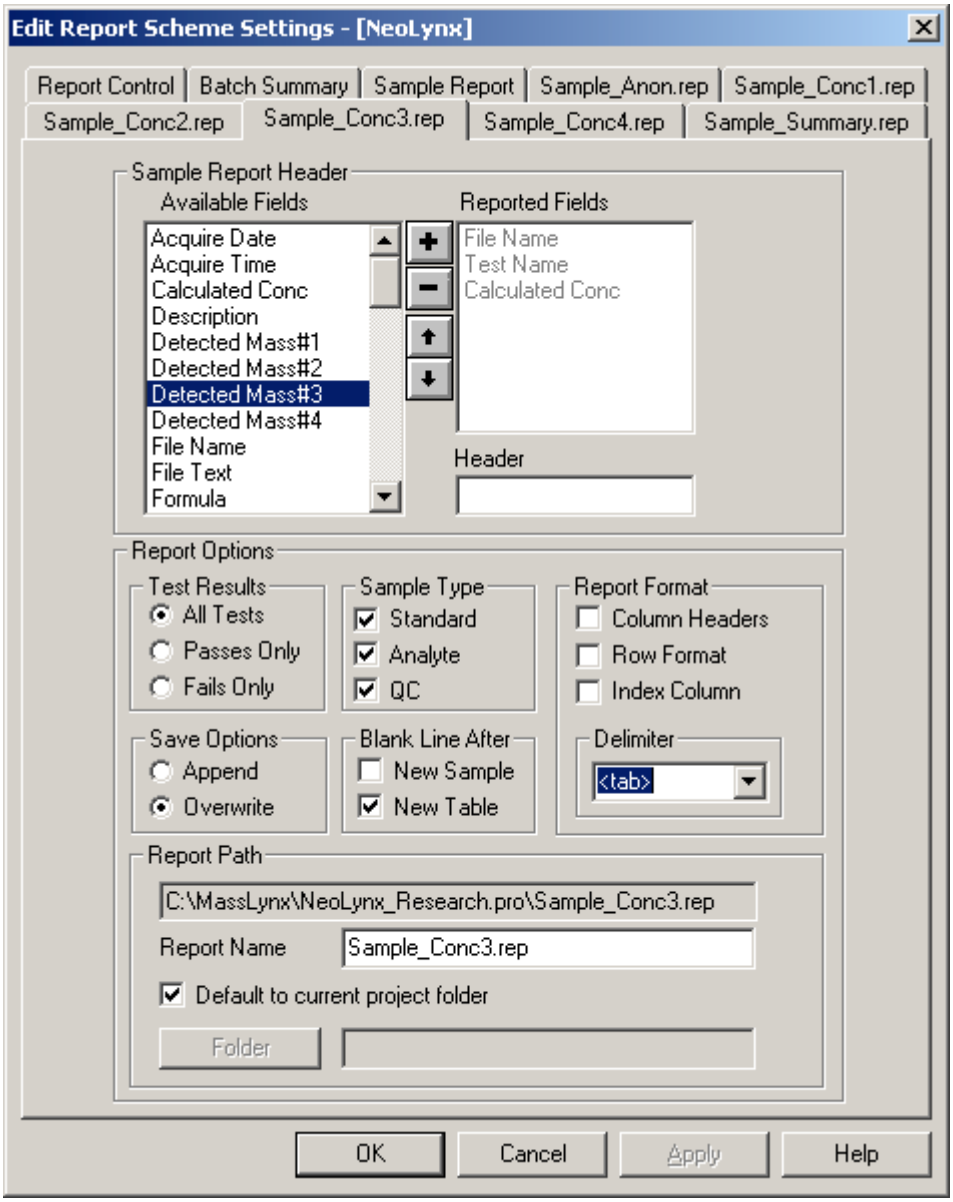
If viewed in a spreadsheet package accepting tab-delimited files, Sample_Conc2.rep will have the appearance:

Sample01	C3	1.51	C4	0.12	C5:1	0.04	C5	0.06	C6	0.3
Sample02	C3	0.89	C4	0.17	C5:1	0.24	C5	0.11	C6	0.17
Sample03	C3	2.07	C4	0.39	C5:1	0.25	C5	0.29	C6	0.23
Sample04	C3	1.68	C4	2.73	C5:1	0.08	C5	0.24	C6	6
Sample05	C3	23.55	C4	0.34	C5:1	0.27	C5	0.33	C6	0.23
Sample06	C3	2.25	C4	0.41	C5:1	0.09	C5	0.49	C6	0.21
Sample07	C3	1.63	C4	0	C5:1	0.34	C5	0.5	C6	0.46
Sample08	C3	0.7	C4	0.22	C5:1	0.08	C5	0.14	C6	0.21
Sample101	C3	1.51	C4	0.12	C5:1	0.04	C5	0.06	C6	0.3
Sample102	C3	0.89	C4	0.17	C5:1	0.24	C5	0.11	C6	0.17
Sample103	C3	2.07	C4	0.39	C5:1	0.25	C5	0.29	C6	0.23
Sample104	C3	1.68	C4	2.73	C5:1	0.08	C5	0.24	C6	6
Sample105	C3	23.55	C4	0.34	C5:1	0.27	C5	0.33	C6	0.23
Sample106	C3	2.25	C4	0.41	C5:1	0.09	C5	0.49	C6	0.21
Sample107	C3	1.63	C4	0	C5:1	0.34	C5	0.5	C6	0.46
Sample108	C3	0.7	C4	0.22	C5:1	0.08	C5	0.14	C6	0.21

Sample01	C5-OH	0.35	C8	0.53	C10:1	0.68	C10	0.37	C14:1	0.17	C16	1.42
Sample02	C5-OH	0.31	C8	0.53	C10:1	0.46	C10	0.6	C14:1	0.25	C16	0.93
Sample03	C5-OH	0.44	C8	0.3	C10:1	0.42	C10	0.38	C14:1	0.22	C16	3.36
Sample04	C5-OH	1.18	C8	34.51	C10:1	3.39	C10	4.18	C14:1	0.23	C16	3.26
Sample05	C5-OH	0.38	C8	0.31	C10:1	0.15	C10	0.37	C14:1	0.59	C16	1.57
Sample06	C5-OH	1.07	C8	0.97	C10:1	0.81	C10	0.68	C14:1	1.81	C16	8.03
Sample07	C5-OH	11.83	C8	1.68	C10:1	1.46	C10	1.67	C14:1	0.89	C16	10.09
Sample08	C5-OH	0.41	C8	0.38	C10:1	0.17	C10	0.41	C14:1	0.2	C16	2.98
Sample101	C5-OH	0.35	C8	0.53	C10:1	0.68	C10	0.37	C14:1	0.17	C16	1.42
Sample102	C5-OH	0.31	C8	0.53	C10:1	0.46	C10	0.6	C14:1	0.25	C16	0.93
Sample103	C5-OH	0.44	C8	0.3	C10:1	0.42	C10	0.38	C14:1	0.22	C16	3.36
Sample104	C5-OH	1.18	C8	34.51	C10:1	3.39	C10	4.18	C14:1	0.23	C16	3.26
Sample105	C5-OH	0.38	C8	0.31	C10:1	0.15	C10	0.37	C14:1	0.59	C16	1.57
Sample106	C5-OH	1.07	C8	0.97	C10:1	0.81	C10	0.68	C14:1	1.81	C16	8.03
Sample107	C5-OH	11.83	C8	1.68	C10:1	1.46	C10	1.67	C14:1	0.89	C16	10.09
Sample108	C5-OH	0.41	C8	0.38	C10:1	0.17	C10	0.41	C14:1	0.2	C16	2.98

Sample01	Leu/Ile	155.06	Met	12.03	Phe	41.7	Tyr	127.64	Phe/Tyr	0.33
Sample02	Leu/Ile	1064.37	Met	6.34	Phe	46.1	Tyr	81.75	Phe/Tyr	0.56
Sample03	Leu/Ile	181.58	Met	10.45	Phe	457.43	Tyr	103.22	Phe/Tyr	4.43
Sample04	Leu/Ile	174.43	Met	14.33	Phe	61.78	Tyr	176.63	Phe/Tyr	0.35
Sample05	Leu/Ile	304.99	Met	15.78	Phe	35.96	Tyr	56.81	Phe/Tyr	0.63
Sample06	Leu/Ile	183.48	Met	12.99	Phe	37.3	Tyr	168.52	Phe/Tyr	0.22
Sample07	Leu/Ile	412.48	Met	15.63	Phe	65.74	Tyr	109.45	Phe/Tyr	0.6
Sample08	Leu/Ile	312.09	Met	13.83	Phe	1264.24	Tyr	87.25	Phe/Tyr	14.49
Sample101	Leu/Ile	155.06	Met	12.03	Phe	41.7	Tyr	127.64	Phe/Tyr	0.33
Sample102	Leu/Ile	1064.37	Met	6.34	Phe	46.1	Tyr	81.75	Phe/Tyr	0.56
Sample103	Leu/Ile	181.58	Met	10.45	Phe	457.43	Tyr	103.22	Phe/Tyr	4.43
Sample104	Leu/Ile	174.43	Met	14.33	Phe	61.78	Tyr	176.63	Phe/Tyr	0.35
Sample105	Leu/Ile	304.99	Met	15.78	Phe	35.96	Tyr	56.81	Phe/Tyr	0.63
Sample106	Leu/Ile	183.48	Met	12.99	Phe	37.3	Tyr	168.52	Phe/Tyr	0.22
Sample107	Leu/Ile	412.48	Met	15.63	Phe	65.74	Tyr	109.45	Phe/Tyr	0.6
Sample108	Leu/Ile	312.09	Met	13.83	Phe	1264.24	Tyr	87.25	Phe/Tyr	14.49

Sample_Conc3.rep



In this format, each line contains a single filename, a single test name and the calculated concentration. The report has been split into columns for ease of viewing. The file is tab-delimited.

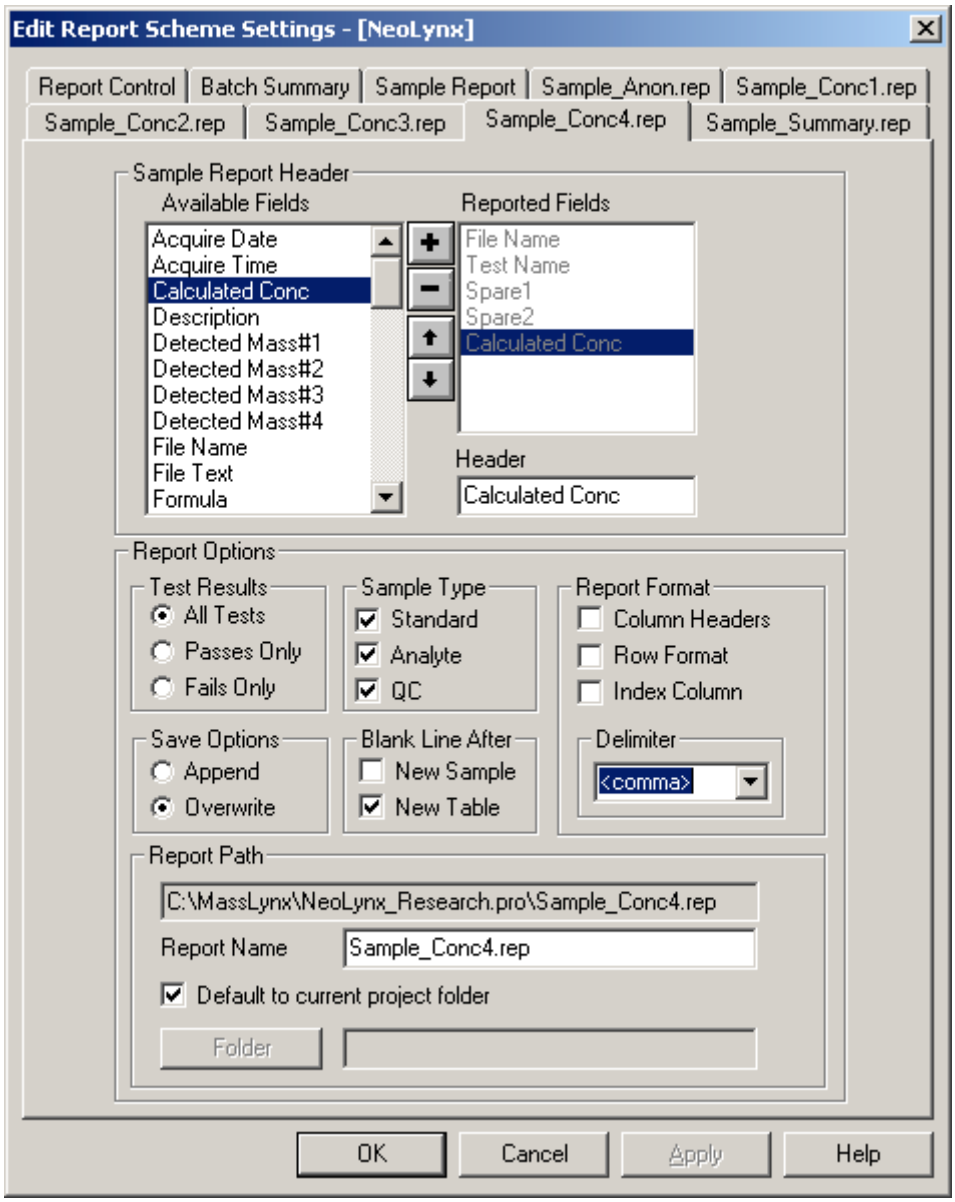
If viewed in a spreadsheet package accepting tab-delimited files, Sample_Conc3.rep will have the appearance:

Sample01	C3 Carnitine	1.51
Sample01	C4 Carnitine	0.12
Sample01	C5:1 Carnitine	0.04
Sample01	C5 Carnitine	0.06
Sample01	C6 Carnitine	0.3
Sample01	C5-OH Carnitine	0.35
Sample01	C8 Carnitine	0.53
Sample01	C10:1 Carnitine	0.68
Sample01	C10 Carnitine	0.37
Sample01	C14:1 Carnitine	0.17
Sample01	C16 Carnitine	1.42
Sample01	Leu/Ile	155.06
Sample01	Met	12.03
Sample01	Phe	41.7
Sample01	Tyr	127.64
Sample01	Phe/Tyr	0.33
Sample02	C3 Carnitine	0.89
Sample02	C4 Carnitine	0.17
Sample02	C5:1 Carnitine	0.24
Sample02	C5 Carnitine	0.11
Sample02	C6 Carnitine	0.17
Sample02	C5-OH Carnitine	0.31
Sample02	C8 Carnitine	0.53
Sample02	C10:1 Carnitine	0.46
Sample02	C10 Carnitine	0.6
Sample02	C14:1 Carnitine	0.25
Sample02	C16 Carnitine	0.93
Sample02	Leu/Ile	1064.37
Sample02	Met	6.34
Sample02	Phe	46.1
Sample02	Tyr	81.75
Sample02	Phe/Tyr	0.56
Sample03	C3 Carnitine	2.07
Sample03	C4 Carnitine	0.39
Sample03	C5:1 Carnitine	0.25
Sample03	C5 Carnitine	0.29
Sample03	C6 Carnitine	0.23
Sample03	C5-OH Carnitine	0.44
Sample03	C8 Carnitine	0.3
Sample03	C10:1 Carnitine	0.42
Sample03	C10 Carnitine	0.38
Sample03	C14:1 Carnitine	0.22
Sample03	C16 Carnitine	3.36
Sample03	Leu/Ile	181.58
Sample03	Met	10.45
Sample03	Phe	457.43
Sample03	Tyr	103.22
Sample03	Phe/Tyr	4.43
Sample04	C3 Carnitine	1.68
Sample04	C4 Carnitine	2.73
Sample04	C5:1 Carnitine	0.08
Sample04	C5 Carnitine	0.24
Sample04	C6 Carnitine	6
Sample04	C5-OH Carnitine	1.18
Sample04	C8 Carnitine	34.51
Sample04	C10:1 Carnitine	3.39
Sample04	C10 Carnitine	4.18
Sample04	C14:1 Carnitine	0.23
Sample04	C16 Carnitine	3.26
Sample04	Leu/Ile	174.43
Sample04	Met	14.33
Sample04	Phe	61.78
Sample04	Tyr	176.63
Sample04	Phe/Tyr	0.35
Sample05	C3 Carnitine	23.55
Sample05	C4 Carnitine	0.34
Sample05	C5:1 Carnitine	0.27
Sample05	C5 Carnitine	0.33
Sample05	C6 Carnitine	0.23
Sample05	C5-OH Carnitine	0.38
Sample05	C8 Carnitine	0.31
Sample05	C10:1 Carnitine	0.15
Sample05	C10 Carnitine	0.37
Sample05	C14:1 Carnitine	0.59
Sample05	C16 Carnitine	1.57
Sample05	Leu/Ile	304.99
Sample05	Met	15.78
Sample05	Phe	35.96
Sample05	Tyr	56.81
Sample05	Phe/Tyr	0.63
Sample06	C3 Carnitine	2.25
Sample06	C4 Carnitine	0.41
Sample06	C5:1 Carnitine	0.09
Sample06	C5 Carnitine	0.49
Sample06	C6 Carnitine	0.21

Sample06	C5-OH Carnitine	1.07
Sample06	C8 Carnitine	0.97
Sample06	C10:1 Carnitine	0.81
Sample06	C10 Carnitine	0.68
Sample06	C14:1 Carnitine	1.81
Sample06	C16 Carnitine	8.03
Sample06	Leu/Ile	183.48
Sample06	Met	12.99
Sample06	Phe	37.3
Sample06	Tyr	168.52
Sample06	Phe/Tyr	0.22
Sample07	C3 Carnitine	1.63
Sample07	C4 Carnitine	0
Sample07	C5:1 Carnitine	0.34
Sample07	C5 Carnitine	0.5
Sample07	C6 Carnitine	0.46
Sample07	C5-OH Carnitine	11.83
Sample07	C8 Carnitine	1.68
Sample07	C10:1 Carnitine	1.46
Sample07	C10 Carnitine	1.67
Sample07	C14:1 Carnitine	0.89
Sample07	C16 Carnitine	10.09
Sample07	Leu/Ile	412.48
Sample07	Met	15.63
Sample07	Phe	65.74
Sample07	Tyr	109.45
Sample07	Phe/Tyr	0.6
Sample08	C3 Carnitine	0.7
Sample08	C4 Carnitine	0.22
Sample08	C5:1 Carnitine	0.08
Sample08	C5 Carnitine	0.14
Sample08	C6 Carnitine	0.21
Sample08	C5-OH Carnitine	0.41
Sample08	C8 Carnitine	0.38
Sample08	C10:1 Carnitine	0.17
Sample08	C10 Carnitine	0.41
Sample08	C14:1 Carnitine	0.2
Sample08	C16 Carnitine	2.98
Sample08	Leu/Ile	312.09
Sample08	Met	13.83
Sample08	Phe	1264.24
Sample08	Tyr	87.25
Sample08	Phe/Tyr	14.49
Sample101	C3 Carnitine	1.51
Sample101	C4 Carnitine	0.12
Sample101	C5:1 Carnitine	0.04
Sample101	C5 Carnitine	0.06
Sample101	C6 Carnitine	0.3
Sample101	C5-OH Carnitine	0.35
Sample101	C8 Carnitine	0.53
Sample101	C10:1 Carnitine	0.68
Sample101	C10 Carnitine	0.37
Sample101	C14:1 Carnitine	0.17
Sample101	C16 Carnitine	1.42
Sample101	Leu/Ile	155.06
Sample101	Met	12.03
Sample101	Phe	41.7
Sample101	Tyr	127.64
Sample101	Phe/Tyr	0.33
Sample102	C3 Carnitine	0.89
Sample102	C4 Carnitine	0.17
Sample102	C5:1 Carnitine	0.24
Sample102	C5 Carnitine	0.11
Sample102	C6 Carnitine	0.17
Sample102	C5-OH Carnitine	0.31
Sample102	C8 Carnitine	0.53
Sample102	C10:1 Carnitine	0.46
Sample102	C10 Carnitine	0.6
Sample102	C14:1 Carnitine	0.25
Sample102	C16 Carnitine	0.93
Sample102	Leu/Ile	1064.37
Sample102	Met	6.34
Sample102	Phe	46.1
Sample102	Tyr	81.75
Sample102	Phe/Tyr	0.56
Sample103	C3 Carnitine	2.07
Sample103	C4 Carnitine	0.39
Sample103	C5:1 Carnitine	0.25
Sample103	C5 Carnitine	0.29
Sample103	C6 Carnitine	0.23
Sample103	C5-OH Carnitine	0.44
Sample103	C8 Carnitine	0.3
Sample103	C10:1 Carnitine	0.42
Sample103	C10 Carnitine	0.38
Sample103	C14:1 Carnitine	0.22
Sample103	C16 Carnitine	3.36
Sample103	Leu/Ile	181.58
Sample103	Met	10.45
Sample103	Phe	457.43
Sample103	Tyr	103.22

Sample103	Phe/Tyr	4.43
Sample104	C3 Carnitine	1.68
Sample104	C4 Carnitine	2.73
Sample104	C5:1 Carnitine	0.08
Sample104	C5 Carnitine	0.24
Sample104	C6 Carnitine	6
Sample104	C5-OH Carnitine	1.18
Sample104	C8 Carnitine	34.51
Sample104	C10:1 Carnitine	3.39
Sample104	C10 Carnitine	4.18
Sample104	C14:1 Carnitine	0.23
Sample104	C16 Carnitine	3.26
Sample104	Leu/Ile	174.43
Sample104	Met	14.33
Sample104	Phe	61.78
Sample104	Tyr	176.63
Sample104	Phe/Tyr	0.35
Sample105	C3 Carnitine	23.55
Sample105	C4 Carnitine	0.34
Sample105	C5:1 Carnitine	0.27
Sample105	C5 Carnitine	0.33
Sample105	C6 Carnitine	0.23
Sample105	C5-OH Carnitine	0.38
Sample105	C8 Carnitine	0.31
Sample105	C10:1 Carnitine	0.15
Sample105	C10 Carnitine	0.37
Sample105	C14:1 Carnitine	0.59
Sample105	C16 Carnitine	1.57
Sample105	Leu/Ile	304.99
Sample105	Met	15.78
Sample105	Phe	35.96
Sample105	Tyr	56.81
Sample105	Phe/Tyr	0.63
Sample106	C3 Carnitine	2.25
Sample106	C4 Carnitine	0.41
Sample106	C5:1 Carnitine	0.09
Sample106	C5 Carnitine	0.49
Sample106	C6 Carnitine	0.21
Sample106	C5-OH Carnitine	1.07
Sample106	C8 Carnitine	0.97
Sample106	C10:1 Carnitine	0.81
Sample106	C10 Carnitine	0.68
Sample106	C14:1 Carnitine	1.81
Sample106	C16 Carnitine	8.03
Sample106	Leu/Ile	183.48
Sample106	Met	12.99
Sample106	Phe	37.3
Sample106	Tyr	168.52
Sample106	Phe/Tyr	0.22
Sample107	C3 Carnitine	1.63
Sample107	C4 Carnitine	0
Sample107	C5:1 Carnitine	0.34
Sample107	C5 Carnitine	0.5
Sample107	C6 Carnitine	0.46
Sample107	C5-OH Carnitine	11.83
Sample107	C8 Carnitine	1.68
Sample107	C10:1 Carnitine	1.46
Sample107	C10 Carnitine	1.67
Sample107	C14:1 Carnitine	0.89
Sample107	C16 Carnitine	10.09
Sample107	Leu/Ile	412.48
Sample107	Met	15.63
Sample107	Phe	65.74
Sample107	Tyr	109.45
Sample107	Phe/Tyr	0.6
Sample108	C3 Carnitine	0.7
Sample108	C4 Carnitine	0.22
Sample108	C5:1 Carnitine	0.08
Sample108	C5 Carnitine	0.14
Sample108	C6 Carnitine	0.21
Sample108	C5-OH Carnitine	0.41
Sample108	C8 Carnitine	0.38
Sample108	C10:1 Carnitine	0.17
Sample108	C10 Carnitine	0.41
Sample108	C14:1 Carnitine	0.2
Sample108	C16 Carnitine	2.98
Sample108	Leu/Ile	312.09
Sample108	Met	13.83
Sample108	Phe	1264.24
Sample108	Tyr	87.25
Sample108	Phe/Tyr	14.49

Sample_Conc4.rep



In this format, each line contains a single filename, a single test name, the value in the Spare1 column in the sample list, the value from the Spare2 column in the sample list (in this case the column is blank) and calculated concentration. The report has been split into columns for ease of viewing. The file is comma-delimited.

If viewed in a spreadsheet package accepting comma-delimited files, Sample_Conc4.rep will have the appearance:

Sample01	C3	0		1.51
Sample01	C4	0		0.12
Sample01	C5:1	0		0.04
Sample01	C5	0		0.06
Sample01	C6	0		0.3

Sample01	C5-OH	0		0.35
Sample01	C8	0		0.53
Sample01	C10:1	0		0.68
Sample01	C10	0		0.37
Sample01	C14:1	0		0.17

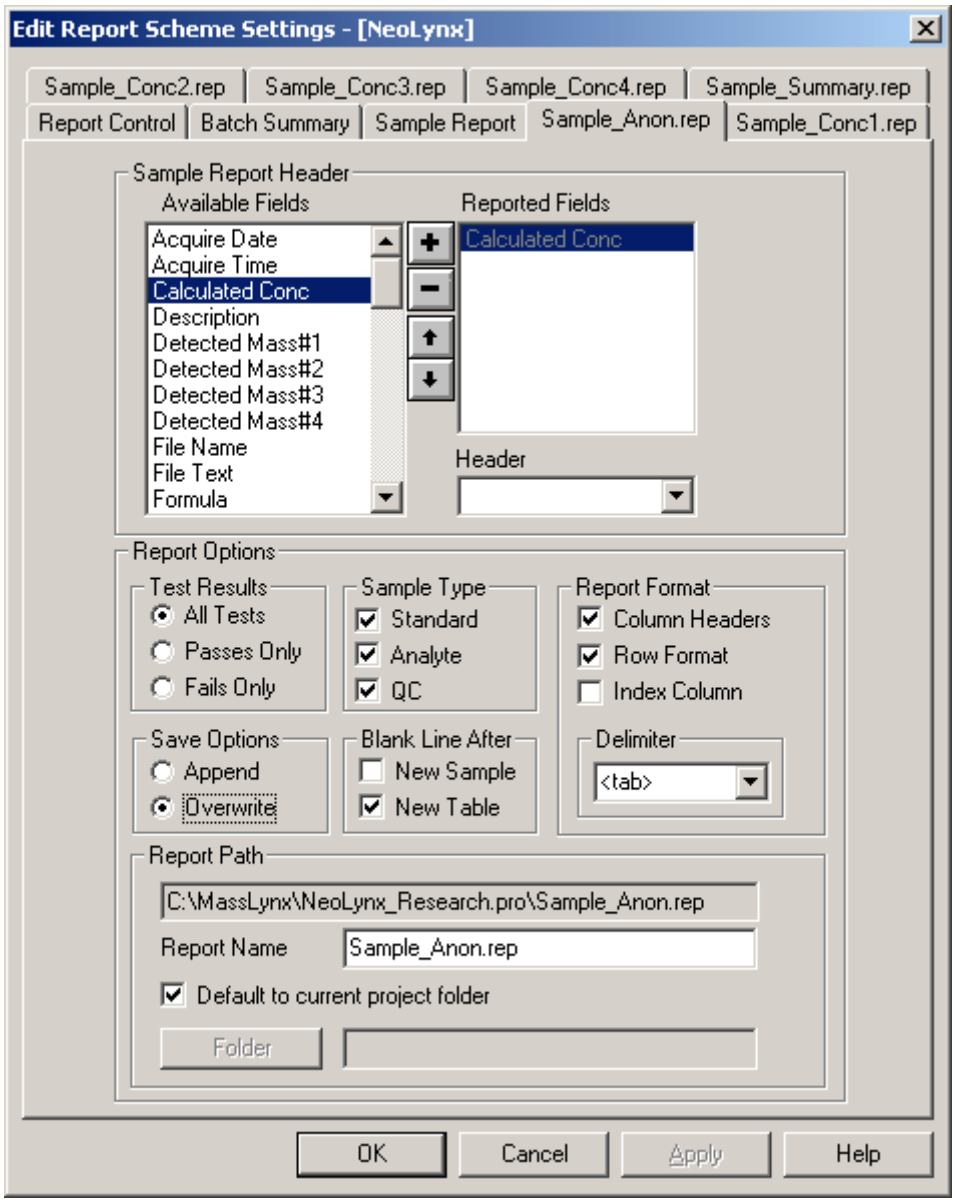
Sample01	C16	0		1.42
Sample01	Leu/Ile	0		155.06
Sample01	Met	0		12.03
Sample01	Phe	0		41.7
Sample01	Tyr	0		127.64
Sample01	Phe/Tyr	0		0.33
Sample02	C3	0		0.89
Sample02	C4	0		0.17
Sample02	C5:1	0		0.24
Sample02	C5	0		0.11
Sample02	C6	0		0.17
Sample02	C5-OH	0		0.31
Sample02	C8	0		0.53
Sample02	C10:1	0		0.46
Sample02	C10	0		0.6
Sample02	C14:1	0		0.25
Sample02	C16	0		0.93
Sample02	Leu/Ile	0		1064.37
Sample02	Met	0		6.34
Sample02	Phe	0		46.1
Sample02	Tyr	0		81.75
Sample02	Phe/Tyr	0		0.56
Sample03	C3	0		2.07
Sample03	C4	0		0.39
Sample03	C5:1	0		0.25
Sample03	C5	0		0.29
Sample03	C6	0		0.23
Sample03	C5-OH	0		0.44
Sample03	C8	0		0.3
Sample03	C10:1	0		0.42
Sample03	C10	0		0.38
Sample03	C14:1	0		0.22
Sample03	C16	0		3.36
Sample03	Leu/Ile	0		181.58
Sample03	Met	0		10.45
Sample03	Phe	0		457.43
Sample03	Tyr	0		103.22
Sample03	Phe/Tyr	0		4.43
Sample04	C3	0		1.68
Sample04	C4	0		2.73
Sample04	C5:1	0		0.08
Sample04	C5	0		0.24
Sample04	C6	0		6
Sample04	C5-OH	0		1.18
Sample04	C8	0		34.51
Sample04	C10:1	0		3.39
Sample04	C10	0		4.18
Sample04	C14:1	0		0.23
Sample04	C16	0		3.26
Sample04	Leu/Ile	0		174.43
Sample04	Met	0		14.33
Sample04	Phe	0		61.78
Sample04	Tyr	0		176.63
Sample04	Phe/Tyr	0		0.35
Sample05	C3	0		23.55
Sample05	C4	0		0.34
Sample05	C5:1	0		0.27
Sample05	C5	0		0.33
Sample05	C6	0		0.23
Sample05	C5-OH	0		0.38
Sample05	C8	0		0.31
Sample05	C10:1	0		0.15
Sample05	C10	0		0.37
Sample05	C14:1	0		0.59
Sample05	C16	0		1.57
Sample05	Leu/Ile	0		304.99
Sample05	Met	0		15.78
Sample05	Phe	0		35.96
Sample05	Tyr	0		56.81
Sample05	Phe/Tyr	0		0.63
Sample06	C3	0		2.25
Sample06	C4	0		0.41
Sample06	C5:1	0		0.09
Sample06	C5	0		0.49
Sample06	C6	0		0.21
Sample06	C5-OH	0		1.07
Sample06	C8	0		0.97
Sample06	C10:1	0		0.81
Sample06	C10	0		0.68
Sample06	C14:1	0		1.81
Sample06	C16	0		8.03
Sample06	Leu/Ile	0		183.48
Sample06	Met	0		12.99
Sample06	Phe	0		37.3
Sample06	Tyr	0		168.52
Sample06	Phe/Tyr	0		0.22
Sample07	C3	A		1.63
Sample07	C4	A		0
Sample07	C5:1	A		0.34
Sample07	C5	A		0.5

Sample07	C6	A		0.46
Sample07	C5-OH	A		11.83
Sample07	C8	A		1.68
Sample07	C10:1	A		1.46
Sample07	C10	A		1.67
Sample07	C14:1	A		0.89
Sample07	C16	A		10.09
Sample07	Leu/Ile	A		412.48
Sample07	Met	A		15.63
Sample07	Phe	A		65.74
Sample07	Tyr	A		109.45
Sample07	Phe/Tyr	A		0.6
Sample08	C3	A		0.7
Sample08	C4	A		0.22
Sample08	C5:1	A		0.08
Sample08	C5	A		0.14
Sample08	C6	A		0.21
Sample08	C5-OH	A		0.41
Sample08	C8	A		0.38
Sample08	C10:1	A		0.17
Sample08	C10	A		0.41
Sample08	C14:1	A		0.2
Sample08	C16	A		2.98
Sample08	Leu/Ile	A		312.09
Sample08	Met	A		13.83
Sample08	Phe	A		1264.24
Sample08	Tyr	A		87.25
Sample08	Phe/Tyr	A		14.49
Sample101	C3			1.51
Sample101	C4			0.12
Sample101	C5:1			0.04
Sample101	C5			0.06
Sample101	C6			0.3
Sample101	C5-OH			0.35
Sample101	C8			0.53
Sample101	C10:1			0.68
Sample101	C10			0.37
Sample101	C14:1			0.17
Sample101	C16			1.42
Sample101	Leu/Ile			155.06
Sample101	Met			12.03
Sample101	Phe			41.7
Sample101	Tyr			127.64
Sample101	Phe/Tyr			0.33
Sample102	C3			0.89
Sample102	C4			0.17
Sample102	C5:1			0.24
Sample102	C5			0.11
Sample102	C6			0.17
Sample102	C5-OH			0.31
Sample102	C8			0.53
Sample102	C10:1			0.46
Sample102	C10			0.6
Sample102	C14:1			0.25
Sample102	C16			0.93
Sample102	Leu/Ile			1064.37
Sample102	Met			6.34
Sample102	Phe			46.1
Sample102	Tyr			81.75
Sample102	Phe/Tyr			0.56
Sample103	C3			2.07
Sample103	C4			0.39
Sample103	C5:1			0.25
Sample103	C5			0.29
Sample103	C6			0.23
Sample103	C5-OH			0.44
Sample103	C8			0.3
Sample103	C10:1			0.42
Sample103	C10			0.38
Sample103	C14:1			0.22
Sample103	C16			3.36
Sample103	Leu/Ile			181.58
Sample103	Met			10.45
Sample103	Phe			457.43
Sample103	Tyr			103.22
Sample103	Phe/Tyr			4.43
Sample104	C3			1.68
Sample104	C4			2.73
Sample104	C5:1			0.08
Sample104	C5			0.24
Sample104	C6			6
Sample104	C5-OH			1.18
Sample104	C8			34.51
Sample104	C10:1			3.39
Sample104	C10			4.18
Sample104	C14:1			0.23
Sample104	C16			3.26
Sample104	Leu/Ile			174.43
Sample104	Met			14.33
Sample104	Phe			61.78

Sample104	Tyr			176.63
Sample104	Phe/Tyr			0.35
Sample105	C3			23.55
Sample105	C4			0.34
Sample105	C5:1			0.27
Sample105	C5			0.33
Sample105	C6			0.23
Sample105	C5-OH			0.38
Sample105	C8			0.31
Sample105	C10:1			0.15
Sample105	C10			0.37
Sample105	C14:1			0.59
Sample105	C16			1.57
Sample105	Leu/Ile			304.99
Sample105	Met			15.78
Sample105	Phe			35.96
Sample105	Tyr			56.81
Sample105	Phe/Tyr			0.63
Sample106	C3			2.25
Sample106	C4			0.41
Sample106	C5:1			0.09
Sample106	C5			0.49
Sample106	C6			0.21
Sample106	C5-OH			1.07
Sample106	C8			0.97
Sample106	C10:1			0.81
Sample106	C10			0.68
Sample106	C14:1			1.81
Sample106	C16			8.03
Sample106	Leu/Ile			183.48
Sample106	Met			12.99
Sample106	Phe			37.3
Sample106	Tyr			168.52
Sample106	Phe/Tyr			0.22

Sample107	C3			1.63
Sample107	C4			0
Sample107	C5:1			0.34
Sample107	C5			0.5
Sample107	C6			0.46
Sample107	C5-OH			11.83
Sample107	C8			1.68
Sample107	C10:1			1.46
Sample107	C10			1.67
Sample107	C14:1			0.89
Sample107	C16			10.09
Sample107	Leu/Ile			412.48
Sample107	Met			15.63
Sample107	Phe			65.74
Sample107	Tyr			109.45
Sample107	Phe/Tyr			0.6
Sample108	C3			0.7
Sample108	C4			0.22
Sample108	C5:1			0.08
Sample108	C5			0.14
Sample108	C6			0.21
Sample108	C5-OH			0.41
Sample108	C8			0.38
Sample108	C10:1			0.17
Sample108	C10			0.41
Sample108	C14:1			0.2
Sample108	C16			2.98
Sample108	Leu/Ile			312.09
Sample108	Met			13.83
Sample108	Phe			1264.24
Sample108	Tyr			87.25
Sample108	Phe/Tyr			14.49

Sample_Anon.rep



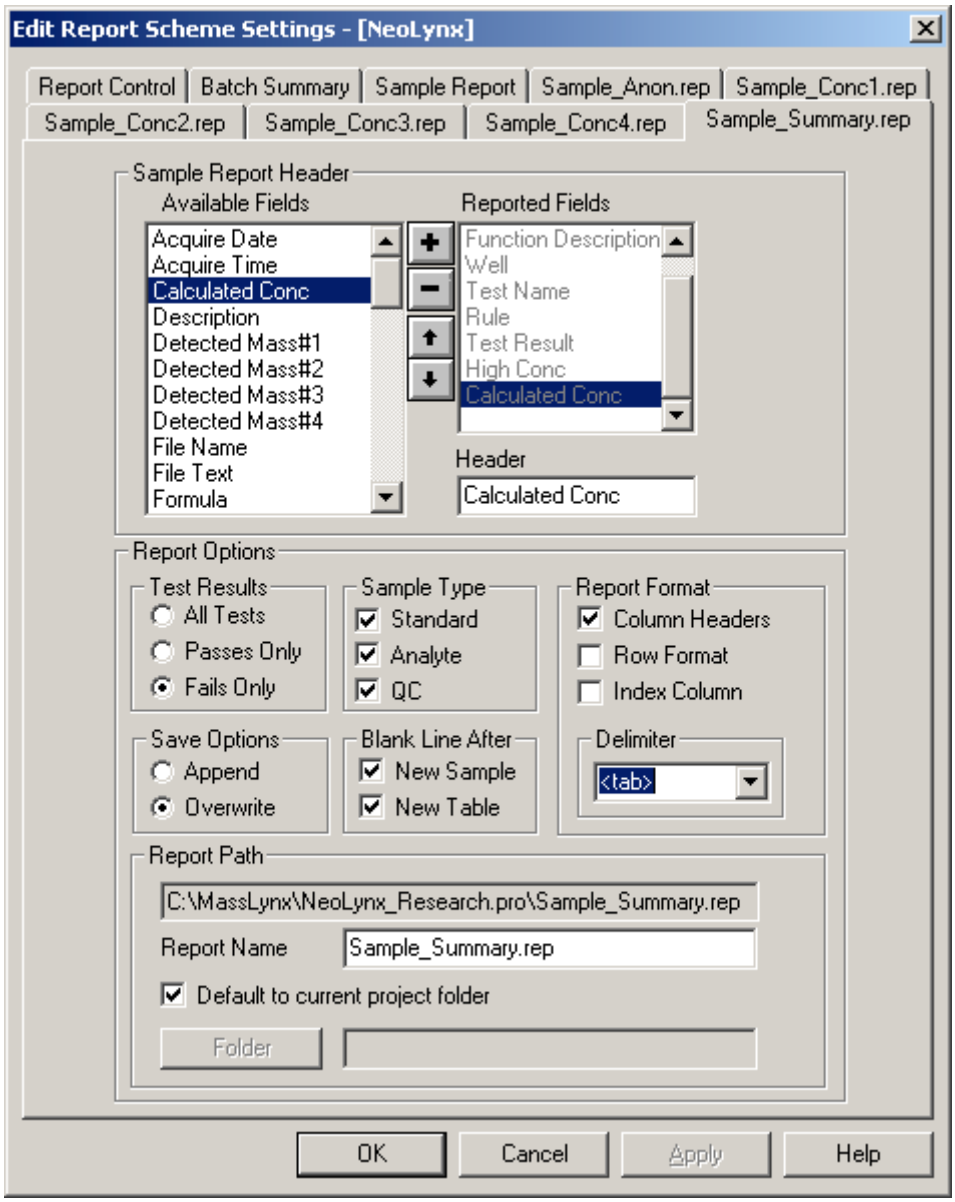
This report contains the same information as Samples_CONC1.REP without the file names. This format is intended for statistical use in population screening. The report has been split into two sections for ease of viewing. The file is tab-delimited.

If viewed in a spreadsheet package accepting tab-delimited files, Sample_Anon.rep will have the appearance:

C3 Carnitine	C4 Carnitine	C5:1 Carnitine	C5 Carnitine	C6 Carnitine	C5-OH Carnitine	C8 Carnitine	C10:1 Carnitine
1.51	0.12	0.04	0.06	0.3	0.35	0.53	0.68
0.89	0.17	0.24	0.11	0.17	0.31	0.53	0.46
2.07	0.39	0.25	0.29	0.23	0.44	0.3	0.42
1.68	2.73	0.08	0.24	6	1.18	34.51	3.39
23.55	0.34	0.27	0.33	0.23	0.38	0.31	0.15
2.25	0.41	0.09	0.49	0.21	1.07	0.97	0.81
1.63	0	0.34	0.5	0.46	11.83	1.68	1.46
0.7	0.22	0.08	0.14	0.21	0.41	0.38	0.17
1.51	0.12	0.04	0.06	0.3	0.35	0.53	0.68
0.89	0.17	0.24	0.11	0.17	0.31	0.53	0.46
2.07	0.39	0.25	0.29	0.23	0.44	0.3	0.42
1.68	2.73	0.08	0.24	6	1.18	34.51	3.39
23.55	0.34	0.27	0.33	0.23	0.38	0.31	0.15
2.25	0.41	0.09	0.49	0.21	1.07	0.97	0.81
1.63	0	0.34	0.5	0.46	11.83	1.68	1.46
0.7	0.22	0.08	0.14	0.21	0.41	0.38	0.17

C10 Carnitine	C14:1 Carnitine	C16 Carnitine	Leu/Ile	Met	Phe	Tyr	Phe/Tyr
0.37	0.17	1.42	155.06	12.03	41.7	127.64	0.33
0.6	0.25	0.93	1064.37	6.34	46.1	81.75	0.56
0.38	0.22	3.36	181.58	10.45	457.43	103.22	4.43
4.18	0.23	3.26	174.43	14.33	61.78	176.63	0.35
0.37	0.59	1.57	304.99	15.78	35.96	56.81	0.63
0.68	1.81	8.03	183.48	12.99	37.3	168.52	0.22
1.67	0.89	10.09	412.48	15.63	65.74	109.45	0.6
0.41	0.2	2.98	312.09	13.83	1264.24	87.25	14.49
0.37	0.17	1.42	155.06	12.03	41.7	127.64	0.33
0.6	0.25	0.93	1064.37	6.34	46.1	81.75	0.56
0.38	0.22	3.36	181.58	10.45	457.43	103.22	4.43
4.18	0.23	3.26	174.43	14.33	61.78	176.63	0.35
0.37	0.59	1.57	304.99	15.78	35.96	56.81	0.63
0.68	1.81	8.03	183.48	12.99	37.3	168.52	0.22
1.67	0.89	10.09	412.48	15.63	65.74	109.45	0.6
0.41	0.2	2.98	312.09	13.83	1264.24	87.25	14.49

Sample_Summary.rep



The Sample_Summary.rep report is probably the most commonly used report format. It shows a summary of all the Tests that have fallen outside the defined limits together with information relating to the sample under analysis. The style of this report has changed slightly from V3.3, but the information content is now considerably more flexible.

If viewed in a spreadsheet package accepting tab-delimited files, Sample_Summary.rep will have the appearance:

File Name	Function Description	Well	Test Name	Rule Name	Result	High Conc	Calc Conc	Units
Sample02	2:Neutral AA	1,1:2	Leu/Ile	1:2 Peak Ratio	High Leu/Ile	400	1064.37	
Sample03	2:Neutral AA	1,1:3	Phe	1:2 Peak Ratio	High Phe	200	457.43	
Sample03	2:Neutral AA	1,1:3	Phe/Tyr	2:4 Peak Ratio	High Phe/Tyr	1.25	4.43	
Sample04	1:Acylcarnitines	1,1:4	C4 Carnitine	1:2 Peak Ratio	High C4	2	2.73	
Sample04	1:Acylcarnitines	1,1:4	C6 Carnitine	1:2 Peak Ratio	High C6	2	6	
Sample04	1:Acylcarnitines	1,1:4	C8 Carnitine	1:2 Peak Ratio	High C8	2	34.51	
Sample04	1:Acylcarnitines	1,1:4	C10:1 Carnitine	1:2 Peak Ratio	High C10:1	2	3.39	
Sample04	1:Acylcarnitines	1,1:4	C10 Carnitine	1:2 Peak Ratio	High C10	2	4.18	
Sample05	1:Acylcarnitines	1,1:5	C3 Carnitine	1:2 Peak Ratio	High C3	20	23.55	
Sample07	1:Acylcarnitines	1,1:7	C5-OH Carnitine	1:2 Peak Ratio	High C5-OH	2	11.83	
Sample07	1:Acylcarnitines	1,1:7	C16 Carnitine	1:2 Peak Ratio	High C16	10	10.09	
Sample07	2:Neutral AA	1,1:7	Leu/Ile	1:2 Peak Ratio	High Leu/Ile	400	412.48	
Sample08	2:Neutral AA	1,1:8	Phe	1:2 Peak Ratio	High Phe	200	1264.24	
Sample08	2:Neutral AA	1,1:8	Phe/Tyr	2:4 Peak Ratio	High Phe/Tyr	1.25	14.49	
Sample102	2:Neutral AA	2,1:2	Leu/Ile	1:2 Peak Ratio	High Leu/Ile	400	1064.37	
Sample103	2:Neutral AA	2,1:3	Phe	1:2 Peak Ratio	High Phe	200	457.43	
Sample103	2:Neutral AA	2,1:3	Phe/Tyr	2:4 Peak Ratio	High Phe/Tyr	1.25	4.43	
Sample104	1:Acylcarnitines	2,1:4	C4 Carnitine	1:2 Peak Ratio	High C4	2	2.73	
Sample104	1:Acylcarnitines	2,1:4	C6 Carnitine	1:2 Peak Ratio	High C6	2	6	
Sample104	1:Acylcarnitines	2,1:4	C8 Carnitine	1:2 Peak Ratio	High C8	2	34.51	
Sample104	1:Acylcarnitines	2,1:4	C10:1 Carnitine	1:2 Peak Ratio	High C10:1	2	3.39	
Sample104	1:Acylcarnitines	2,1:4	C10 Carnitine	1:2 Peak Ratio	High C10	2	4.18	
Sample105	1:Acylcarnitines	2,2:5	C3 Carnitine	1:2 Peak Ratio	High C3	20	23.55	
Sample107	1:Acylcarnitines	2,2:7	C5-OH Carnitine	1:2 Peak Ratio	High C5-OH	2	11.83	
Sample107	1:Acylcarnitines	2,2:7	C16 Carnitine	1:2 Peak Ratio	High C16	10	10.09	
Sample107	2:Neutral AA	2,2:7	Leu/Ile	1:2 Peak Ratio	High Leu/Ile	400	412.48	
Sample108	2:Neutral AA	2,2:8	Phe	1:2 Peak Ratio	High Phe	200	1264.24	
Sample108	2:Neutral AA	2,2:8	Phe/Tyr	2:4 Peak Ratio	High Phe/Tyr	1.25	14.49	

Appendix F Internal Standard Methodology

By far the most efficient way to enhance the accuracy of an assay is by the addition of an internal standard to the entire sample. The sample is then mixed to assure homogeneity during aliquoting. The internal standard is chosen such that identical or very similar behavior during the various phases of the analytical process is guaranteed. Isotopically labeled analogues make the most ideal internal standards and mass spectrometers allow ready differentiation from the natural compound. Concentration calculations are then based on the relative response for the analyte to that of the internal standard and contributions due to matrix, recovery or aliquoting will cancel. The relative response, of the analyte to the internal standard must be known and is usually expressed in terms of a response factor or determined in situ.

The response factor is defined as that number by which the response of the analyte must be multiplied to equal the response of the internal standard, present in equal concentration.

If the response factor used has been determined externally, then the accuracy of the final result depends on the certainty with which the concentration of the internal standard in the analytical mixture is known. Response factors can be determined in situ by the use of calibration lines or single point calibrations, if linearity is assured. In that case, accuracy of the calculated results only depends on the error in aliquoting the internal standard; the actual concentration of the internal standard cancels during the mathematical manipulations. The latter method is preferred in situations where internal standard preparations have to be frequently replenished. The internal standard is prepared at the desired approximate concentration and then aliquoted into each sample with a high precision devise. Again, the exact volume dispensed is not critical as long as the volumes delivered are identical for each dispensation used for calibrators and for samples.

Equations governing internal standard methodology.

[ ] = concentration

R = response

U = unknown or analyte

IS = internal standard

RF = response factor

Equation. 1: $[U] = R_U/R_{IS} \times [IS] \times RF_U$ and

Equation. 2: $RF_U = R_{IS}/R_U \times [U]/[IS]$

Determination of response factor:

Prepare several samples of different analyte concentration. To each sample add an equal aliquot of internal standard and mix to homogeneity. Analyze each sample in triplicate and plot the response ratio, R_U/R_{IS} vs. the concentration of the analyte, [U]. Analyze the plot for linearity.

In linear systems, calculate response factor. RF is the inverse of the slope of the line divided by the internal standard concentration.

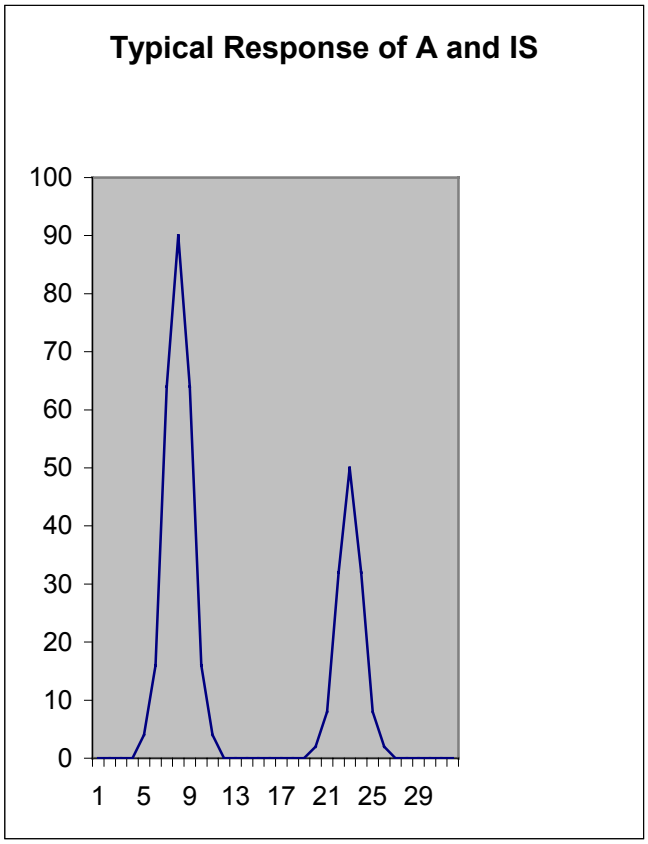
Equation 1 can be restated as:

Equation. 3: $[U] = R_U/R_{IS} \times \text{constant}$

If the response factor is determined from in-run calibrators and the line goes through zero, then

Equation. 4: $[U]_{Sample} = \{ (R_U/R_{IS})_{Sample} / (R_U/R_{IS})_{Calibrator} \} \times [U]_{Calibrator}$

Hence the internal standard concentration cancels.



Index