Education and Culture

Erasmus Mundus

UNIVERSITAT DE BARCELONA

UNIVERSITAT DE BARCELONA
UNIVERSITY OF BARCELONA

FACULTAT DE QUÍMICA
FACULTY OF CHEMISTRY

Development and Application of Quality Standard Procedures (Operation, Verification and Maintenance) for an LC-MS System

EUROPEAN MASTER IN QUALITY IN ANALYTICAL LABORATORIES

emQal

CHRISTY SARMIENTO DANIEL

Barcelona, February 2010

The current work titled

"Development and Application of Quality Standard Procedures (Operation, Verification and Maintenance) for an LC-MS System"

has been conducted by Christy Sarmiento Daniel in the Analytical Chemistry Department of University of Barcelona, Spain.

Dr. Javier Santos	Dr. Oscar Núñez

Barcelona, February 2010

ACKNOWLEDGEMENT

I would like to give my special thanks to all the persons, who are in one way or another, had helped me finished this master thesis. To:

My supervisors, Dra. Encarnacion Moyano, Dr. Javier Santos and Dr. Oscar Núñez, for their guidance and persistent help; for sharing with me their expertise and research insights;

CECEM Head, Dra. Maria Teresa Galceran, for her kind regards; CECEM research group (Ana, Elida, Lorena, Xavi, Jorge, Eloy, Hector, Soubhi and Sarah) for the friendly support and encouragement; for the knowledge and skills I have learned;

The funding institution and organizing body of this master program: European Union and Erasmus Mundus for sharing their capacities to deserving individuals like me;

EMQAL Coordinator, Prof. Isabel Cavaco, for her guidance and support;

My fellow EMQAL students for the unique experiences and friendship;

My Barcelona housemates (Rami, Marta, Jo, Rufie and adopted housemate Jelena) for their camaraderie;

All my friends and relatives for your faith and prayers;

My parents, my brother Alvin and my sister Anne for continuously showering me their love and support in spite of distance; and

Our Almighty God for giving me strength to live all the challenges in my life.

Christy S. Daniel

I N D E X

ABSTRACT

In this work, the standard procedures required for the operation, verification and maintenance of a liquid chromatography coupled to mass spectrometry system have been developed. These procedures have been designed and prepared with the aim to establish a quality control system to ensure the proper functioning of each component of the instrumentation, the LC and the MS, and to verify the performance of the LC-MS coupling. For this purpose, standard procedures were elaborated and proved in the normal routine laboratory work to evaluate their real applicability. Moreover, the verification of the performance of the LC-MS system was carried out experimentally through an in-house procedure based on the analysis of naphthylacetics.

0. OBJECTIVES

The main objective of this work was the development of a quality system for an LC-MS instrument used in a research laboratory. In order to achieve this main objective, the following activities were carried out:

a. verification of the performance of an HPLC instrument;

b. calibration and verification of a mass spectrometer;

c. verification of the performance of an LC-MS system by determination of quality parameters (limit of detection, limit of quantification, linearity and precision) for LC-MS/MS analysis of naphthylacetics;

d. preparation of documents necessary for carrying out instrument operation, maintenance and verification for HPLC, MS and the LC-MS coupling.

1. INTRODUCTION

1.1. Quality assurance and quality control in analytical laboratories

Analytical laboratories have the desire to produce quality results since chemical measurements have great impact on the functioning of a society such as in the areas of forensic analysis, trade, environmental monitoring, and healthcare, among others. By producing valid, reliable and traceable analytical results, the laboratory is benefited by the mutual acceptance of the data by manufacturers, regulators, traders and governments on national and international levels. Moreover, laboratories producing valid measurement data have a higher status in the analytical world which makes them competitive in an open market (Prichard and Barwick, 2007).

Quality assurance and quality control are component´s of the laboratory´s quality management system. The International Organization for Standardization (ISO) (2005) defines quality assurance (QA) as "part of quality management focused on providing confidence that quality requirements will be fulfilled". These are the overall measures taken by the laboratory to ensure and monitor quality.

At present, there are a number of standards dealing with quality assurance: (a) ISO 9001:2000, Quality Management Systems – Requirements; (b) ISO 17025:2005, General Requirements for the Competence of Testing and Calibration Laboratories; (c) ISO 15189:2003, Medical Laboratories – Particular Requirements for Quality and Competence; and (d) GLP, Good Laboratory Practice (GLP). However, these quality assurance systems only provide the general guidelines on how to implement and maintain a given quality system. The implementation of a quality system is a voluntary process and it is the responsibility of the laboratory to define the appropriate procedures necessary to assure that an adequate quality is achieved (Masson, 2007).

ISO (2005) defines quality control as "part of quality management focused on fulfilling quality requirements". It is the planned activities designed to verify the quality of the measurements. Quality control can be internal or external. Internal quality control involves the operations carried out by the laboratory staff as part of the measurement process which provides evidence that the system is operating satisfactorily

with acceptable results. External quality control, on the other hand, provides confidence that the laboratory´s performance is comparable with other laboratories. In order to achieve this, the laboratory participates in formal (proficiency testing schemes) or informal intercomparison exercises (Prichard and Barwick, 2007).

The CITAC/Eurachem Guide (1999) to quality in analytical chemistry cites that the laboratories must operate an appropriate level of internal QC checks and participate in appropriate proficiency testing schemes as part of their quality system and monitoring of day-to-day and batch-to-batch analytical performance. The degree of quality control that needs to be carried out depends on the nature of the analysis, the frequency of analysis, the batch size, the degree of automation, and the test difficulty and reliability. Typical measures includes (a) analysis of reference materials/measurement standards, (b) analysis of blind samples, (c) use of QC samples and control charts, (d) analysis of blanks, (e) replicate analysis, and (f) proficiency testing (Simonet, 2005).

In laboratories, the quality processes that are implemented should demonstrate that the analytical method and instrument provide accurate and precise results. With this regard, a quality procedure should include tests which provide information on the performance characteristics of the method and the instrument and set performance criteria to assist in evaluating the said performance characteristics.

1.2. Implementation of the analytical quality control system

Over the past years, liquid chromatography coupled to mass spectrometry (LC-MS) has become a routine method for many analytical determinations. Highly specific requirements are imposed on these methods to assure that the results obtained are reliable, with high accuracy and precision. Both the method and the instrument contribute to the quality of the results and for these reasons it is necessary to check whether the instrument and the method meets the demands made on the analytical system through validation.

According to the ISO 9000 standard series (2005), validation is the "confirmation, through the provision of the objective evidence, that requirements for a

specific intended use or application have been fulfilled". It provides documented evidence that an instrument, a system, a method or a procedure performs as expected within the specified parameters and requirements to ensure that the results obtained are reliable. Validation efforts should address both the instrument and the computer controlling it and the analytical method run on that equipment. Finally, after these had been verified, they should be checked together (normally in a form of a system suitability test) to confirm the overall performance limits.

The need for validation may originate from regulations and accreditation standards but this is also a prerequisite in terms of any good analytical practice. Validation is a regular process that consists of at least three stages: (1) equipment validation/qualification, (2) analytical method validation, and (3) analytical system suitability test (SST) (Papadoyannis and Samanidou, 2005).

1.2.1. Equipment validation/qualification

Equipment qualification is one of the first steps in analytical method validation and it is a formal systematic process that provides confidence and documented evidence that an instrument is fit for its intended purpose and kept in a state of maintenance and calibration consistent with its use. Qualification is not a single, continuous process but is a result of many discrete activities which have been grouped into four phases: design qualification (DQ), installation qualification (IQ), operational qualification (OQ) and performance qualification (PQ). A typical qualification process is shown in Figure 1.1 (Smith, 2007). Qualification is performed via documented procedures which contains the specific instructions and acceptance criteria that need to be executed and met (Bedson and Rudd, 1999).

Design qualification covers all the procedures prior to the installation of the system in the selected environment. This is the `planning part´ of the EQ process where user requirements specifications and the details for purchasing the equipment are defined (Bedson and Rudd, 1999). Typically DQ includes: (a) description of the intended use of the equipment; (b) selection of the analysis technique, of the technical, environmental and safety precautions, final selection of the supplier and of the

equipment; and (c) development and documentation of final functional and operational specifications (Papadoyannis and Samanidou, 2005).

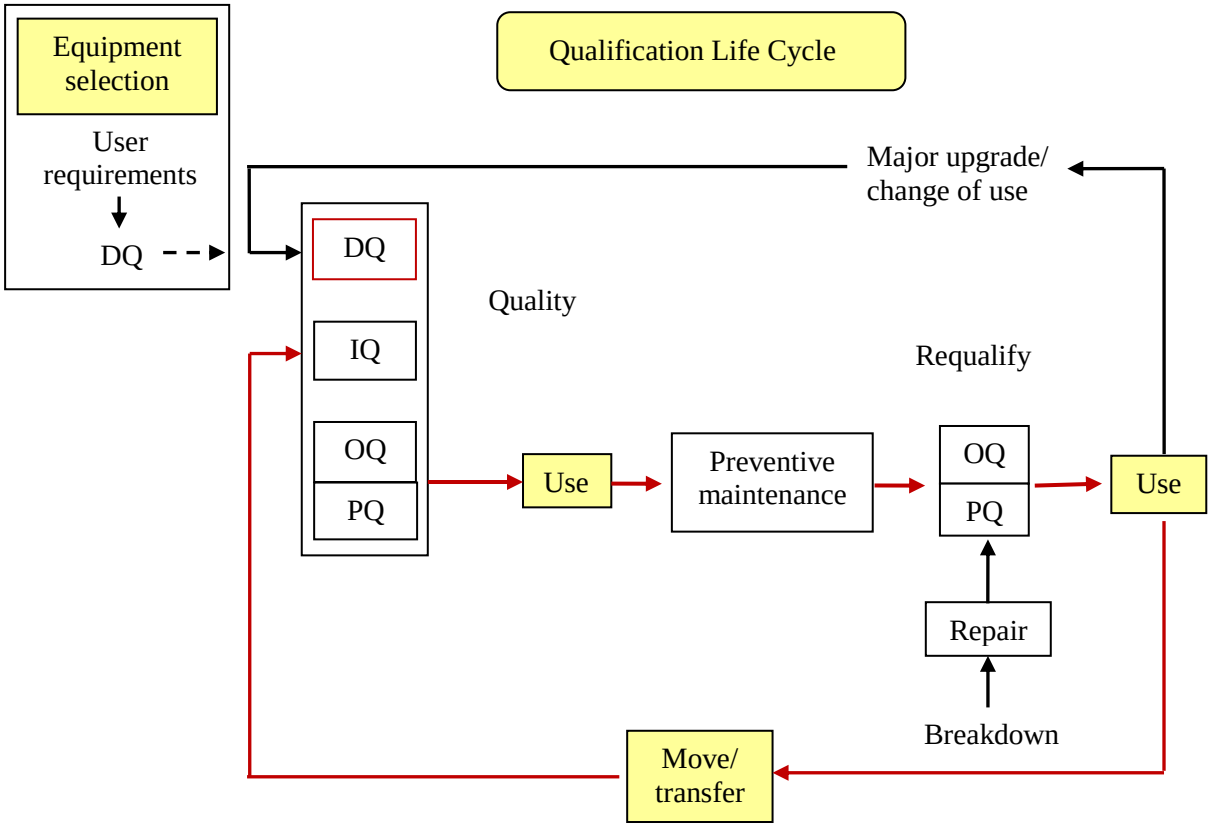

Figure 1.1. A typical qualification lifecycle (Smith, 2007).

Installation qualification is a process used to establish that the instrument was received as specified and installed correctly according to the design requirements in an environment suitable for its operation. Proper installation ensures proper functioning of the equipment.

Operational qualification verifies the key aspects of instrumental performance in the absence of any contributory effects which may be introduced by the method. This involves checking the performance of individual instrument modules to provide evidence that they are operating correctly and within specification. Operational qualification should be carried out after the initial installation of the equipment and must be repeated at regular stages during the life of the instrument depending on the

manufacturer´s recommended intervals, the required performance of the instrument, the nature and usage of the instrument, the environmental conditions where the instrument is installed and the time that the instrument performance is operating under control. In some instances, event-driven OQ is repeated whenever there is (a) routine maintenance, servicing and replacement of parts, (b) movement or relocation, (c) interruption to services and/or utilities, (d) modification or upgrades, (e) troubleshooting/faultfinding after PQ failure.

Performance qualification (PQ) documents the performance of the instrument on continuous operation. It can be considered as having two stages: (1) an initial PQ which is performed after OQ in order to verify the overall performance of the system via a holistic test which involves analyzing a test mixture on a test column; and (2) an ongoing PQ (system suitability checking) to provide continued evidence of the suitability of the instrument´s performance (Bedson and Rudd, 1999). In the event that PQ fails to meet the specifications, the instrument requires maintenance or repair or calibration and the relevant PQ test(s) should be repeated to ensure that the instrument remains qualified. In all these undertakings, standard operating procedures must be maintained and all the activities are recorded (Bansal, et.al., 2004).

1.2.1.1. Instrument maintenance, calibration and verification

Laboratory instrument has to be maintained on a regular basis in order to avoid system failure during operation. Its performance must be reviewed on a regular basis in order to ensure that the instrument is reliable and continues to comply with the requirements specified by the user. Proper maintenance not only makes sense from a scientific point of view but also for financial reasons. Any routine maintenance procedure suggested by the vendor should be followed. The laboratory can also establish its own set of maintenance procedures based upon how the instrument is being used, the types of samples run, and the number of users (considering their level of training and expertise) that have access to the instrument.

Section 5.5.2 of the ISO 17025 standard (2005) states that "…Calibration programmes shall be established for key quantities or values of the instruments where

these properties have a significant effect on the results. Before being placed into service, equipment (including that used for sampling) shall be calibrated or checked to establish that it meets the laboratory´s specifications requirements and complies with relevant standard specifications. It shall be checked and/or calibrated before use." Hence, for laboratories adopting a quality system it is necessary that instrument calibration and verification are put into practice.

Calibration and verification are two terms that are often used incorrectly but each has distinct meaning. ISO/IEC Guide 99 (2007) defines calibration as the "operation that, under specified conditions, in a first step, establishes a relation between the quantity values with measurement uncertainties provided by measurement standards and corresponding indications with associated measurement uncertainties and, in second step, uses this information to establish a relation for obtaining a measurement result from an indication." Calibration is also used to describe the process where several measurements are necessary to establish the relationship between response and concentration which results to the generation of a calibration graph (Prichard and Barwick, 2007).

Verification, on the other hand, is defined by ISO/IEC Guide 99 (2007) as the "provision of evidence that a given item fulfills specified requirements" - Performance verification of an analytical instrument involves comparison of the test results with specifications. It includes testing and requires the availability of clear specifications and acceptance criteria. Calibration permits the estimation of errors of the measuring instrument or the assignment of values to marks on arbitrary scales, whereas, verification of an instrument provides a means of checking whether the deviations between the values indicated by the instrument and the known values of a measured quantity are acceptable or not (Prichard and Barwick, 2007).

The instrument performance will continue to deteriorate through time due to wear and ageing of the components. While routine maintenance can counteract this reduced performance in a short term, it will be inevitable in the long run. However, it is necessary to establish that the instrument continuously meets the minimum established criteria or acceptance limits. These acceptance criteria should not exceed more than what is appropriate for the actual needs of the laboratory otherwise if the established

acceptance criteria are unnecessarily high then it will be difficult to maintain the instrument "within specification". In common applications, when confronted with having more than one instrument of the same type but are from different manufacturers and having different ages, the performance testing process is simplified by choosing less stringent common acceptance criteria that all of the instruments can meet (Currell, 2000).

1.2.1.2. LC-MS performance verification

For an LC-MS system, it is necessary to verify the performance of LC and the MS separately. Likewise, it is important that the coupling of these two instruments demonstrates satisfactory performance. The complexity of the instrumentation often dictates the level of verification necessary to be performed. This is the case with hyphenated instruments such as the LC-MS especially when the individual systems come from different vendors. Normally, the performances of the individual systems (the LC and the MS) are readily verified according to each of the vendor´s procedure. However, when dealt with a coupled system such as LC-MS, the verification of its performance as a whole system can be a quite complicated process. The laboratory is responsible for ensuring that the performance of the LC-MS is still under quality control. To achieve this purpose, the laboratory can choose to develop its own verification process that is scientifically sound, straightforward to use and adequate for the intended application. In this context, it is proposed to use a method with known performance characteristics in order to verify the whole LC-MS system.

1.2.1.2.1. HPLC performance verification

The performance of an HPLC system can be evaluated by examining the key attributes of the various modules comprising the system, followed by a holistic test that takes into account performance of the integrated system as a whole. According to Lam (2004), these are the key performance attributes of the HPLC modules that are checked:

a) Pump module – flow rate accuracy, gradient accuracy and precision, pressure test

b) Injector module – precision, linearity, carryover

c) UV-Visible detector module – wavelength accuracy, linearity of response, noise and drift

d) Column heating module – temperature accuracy and temperature stability

After a verification test, the results are assessed in terms of the predefined acceptance criteria. These criteria had been defined from previously set user requirements. Whenever failure is indicated after the performance verification tests, an impact assessment should be made to evaluate the effect of the failure on the quality of the data generated by the system.

1.2.1.2.2. Column performance verification

The chromatographic column influences the effective separation of the analytes in a given sample. Before a column is purchased, it is necessary to obtain some information regarding column specifications and performance characteristics which are valuable for method development and routine use. The quality or performance of the column deteriorates through time depending on how the operator uses it. Eventually, an HPLC column will decrease its efficiency hence it is important to monitor its performance. The following parameters are normally determined in a given test compound: (a) number of plates (N), (b) peak tailing factor (or symmetry factor) and (c) capacity factor (k´).

The plate number (N) measures the ability of a column to produce a peak that is narrow in relation to its retention time. It is generally estimated from a peak (a neutral compound) which appears towards the end of the chromatogram in order to get a reference value. It is dependent on the chosen solute and the operational conditions adopted.

Symmetrical peaks are always preferred since peaks with poor symmetry can result to inaccurate measurements of plate number and resolution, imprecise quantitation, poor resolution leading to undetected minor bands in the peak tail a poor reproducibility of retention times (Snyder, et.al., 1997). The quality of the peak shape is measured in terms of the tailing factor (Tf).

The capacity factor or retention factor (k´) is a measure of the time the sample component resides in the stationary phase relative to the time it resides in the mobile phase; it expresses how much longer a sample component is retarded by the stationary phase than it would take to travel through the column with the velocity of the mobile phase (IUPAC, 1993).

1.2.1.2.3. Mass spectrometer performance verification

The satisfactory performance of the mass spectrometer depends on its calibration and proper functioning of the instrument system such as electronics and vacuum systems, among others. Normally, an MS instrument has built-in options for checking the overall condition of the instrumental system.

The mass spectrometer provides accurate measurement only if the *m/z* axis is properly calibrated. The calibration is performed using automated procedures often included in the instrument software. During the calibration procedure, a mixture of a MS calibrants (well-characterized reference compounds) are introduced in the ion source of the mass spectrometer, ionized and monitored their spectrum. The calibration of the *m/z* axis can be performed by comparing the theoretical and the experimental spectrum of the reference compound.

A calibration standard mass must have the following characteristics: (a) it should yield a sufficient number of regularly spaced abundant ions across the entire mass scan range; and (b) it should be chemically inert (Dass, 2007). There are several compounds that were proposed to be used as calibration standards in electrospray LC-MS. The proposed calibrants include (a) cesium iodide or cesium carbonate cluster ions, (b) poly(ethylene glycol) (PEG) and poly(propropylene glycol) (PPG), (c) proteins such as the peptide MRFA and myoglobin, (d) Ultramark 1621, a mixture of fluorinated phosphazenes, (e) water cluster ions and (f) sodium trifluoroacetate cluster ions (Niessen, 2006).

1.2.1.2.4. System Suitability Test

The system suitability test for a method is based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such (ICH, 2005). The parameters necessary to be established for system suitability test will depend on the particular method being tested. The parameters and the criteria must be carefully chosen so as to provide unbiased results. System suitability tests are usually done at the start of the analysis but depending on the length of the run or the importance of the sample results, system suitability test may also be performed during and following the analysis (Wells and Dantus, 2005).

1.2.1.2.5. Analytical method performance characteristic determination

A newly developed analytical method must at least provide some analytical figures of merit or performance characteristics for future reference to other analysts that will adopt the method in the future (Krull and Swartz, 1999).

The typical method characteristics that need to be evaluated are: selectivity/specificity, accuracy, precision (repeatability, intermediate precision), limit of detection (LOD) or detection limit, limit of quantification (LOQ) or quantification limit, and linearity and linear range. These definitions are in accordance with the ICH Harmonized Tripartite Guideline for the Validation of Analytical Procedures (2005):

a) Specificity – the ability of to assess unequivocally the analyte in the presence of other components which may be expected to be present.

b) Accuracy – expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

c) Precision – expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

d) Limit of detection – the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

e) Limit of quantification – the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

f) Linearity – the ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

g) Linear range – the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

The method´s performance characteristics should be based on the intended use of the method and the requirements may need to be assessed depending upon the nature of the method and test specifications.

1.3. Documentation in laboratory practice

Documentation is a critical part of a quality assurance system. Laboratories should maintain and control documents related to sampling procedures, calibration procedures, analytical and test methods, data collection and reporting procedures, auditing procedures and checklists, sample handling and storage procedures, computation and data validation procedures, quality assurance manuals, quality plans, sampling data sheets and specifications (Ratliff, 2003).

Standards and regulations require that the laboratory should have written, clear and detailed procedures for all the activities that are performed in the laboratory. A standard operating procedure (SOP) describes the set of instructions a technician or an analyst follows when carrying out an analysis or a process (Kenkel, 2000). SOP forms part of the hierarchy of quality documentation. Several advantages can be cited for having a readily accessible, user-friendly, agreed set of SOPs: they provide evidence that all procedures are in place; they reflect the laboratory´s commitment to quality

standards; they ensure standardization of procedures; they reduce variability and errors; they provide an invaluable platform for staff training and support (Carson and Dent, 2007).

2. EXPERIMENTAL

These were the standards, the reagents and the instrumentation used for carrying out the different verification processes.

2. 1. Chemicals and Instrumentation

2.1.1. Standards and Reagents

HPLC performance verification

- Caffeine, Merck

Column verification

- Uracil, 98%, Merck
- Acetophenone, 99%, Sigma, USA
- Toluene, GC grade, Merck

LCQ MS calibration

- Caffeine, Sigma
- MRFA (L-methionyl-arginyl-phenylalanyl-alanine acetate·H_2O), Sigma
- Ultramark 1621, Sigma

HPLC-MS analysis

- 1-naphthylacetamide PESTANAL®, 99%, Sigma-Aldrich
- 1-naphthoxyacetic acid, 98%, Aldrich
- 2-naphthoxyacetic acid PESTANAL®, 98%, Sigma-Aldrich

HPLC performance verification

- Water , LC-MS grade, Fluka Sigma Aldrich
- Methanol, LC-MS grade, Fluka Sigma Aldrich
- Acetonitrile, LC-MS grade, Fluka Sigma Aldrich
- Acetone, GC grade, Merck

Column verification

- Water, LC-MS grade, Fluka Sigma Aldrich
- Acetonitrile, LC-MS grade, Fluka Sigma Aldrich

LCQ MS calibration

- Water, LC-MS grade, Fluka Sigma Aldrich

- Methanol, LC-MS grade, Fluka Sigma Aldrich
- Acetonitrile, LC-MS grade, Fluka Sigma Aldrich

HPLC-MS analysis

- Methanol, LC-MS grade, Fluka Sigma Aldrich
- Water, LC-MS grade, Fluka Sigma Aldrich

for mobile phase preparation

- Glacial acetic acid, analytical grade, Merck

2.1.2. Instrumentation

2.1.2.1. Liquid chromatography

- Dionex HPLC system (Dionex Softron GmbH, Germany) which consists of
  - SOR-100A-6 solvent rack
  - P680 A DGP-6 high-precision gradient pump with 3 solvent channels each for the left and right pump
  - ASI-100 automated sample injector
  - TCC-100 thermostatted column compartment
  - UVD 170U UV-Vis diode array detector
  - Chromeleon 6.70 chromatography management software

- Accela HPLC system (Thermo Electron San Jose, USA) which consists of
  - quarternary pump with vacuum degasser
  - autosampler which also includes the column oven tray compartment heater/cooler
  - PDA detector
  - Xcalibur data system

- Ascentis® Express RP-Amide, 10cm x 2.1 mm, 2.7 μm (Supelco)

- LiChrospher 100 RP-18 (5μm) HPLC cartridge, 125mm x 4mm (Agilent)

2.1.2.2. Mass spectrometry

Finnigan LCQ quadrupole ion trap mass spectrometer (Thermo Electron San Jose, USA):

- electrospray (ESI) and atmospheric pressure chemical ionization (APCI) sources
- ion trap mass analyzer
- Xcalibur® data system

2.1.2.3. Other instruments

- Analytical balance (Mettler Toledo AT261 Delta Range)
- Calibrated digital thermometer with thermal probe (Testo 945), calibrated at 0 to 200°C by ENAC

2.2. Experimental Procedure

This section describes the different activities that were performed in order to accomplish the objectives of the study. The different procedures for the performance verification of the HPLC, the column, the mass spectrometer and the LC-MS are presented.

2.2.1. HPLC performance verification

The detailed procedure for the performance verification of the Dionex HPLC-UV-Vis is described in the document *Instructions for the Performance Verification of the Dionex HPLC System with UV/Vis Diode Array Detector* (SOP/CECEM/EQP/02/01). The verification included the determination of the performance attributes of the different HPLC modules: pump, autosampler, column oven and detector.

The verification of the Dionex HPLC-UV was performed using a LiChrospher 100 RP-18 column, 125mm x 4mm, 5µm (Agilent) instead of a restriction capillary. The column oven temperature was set to 25°C, except in column oven verification. The column and the system were allowed to equilibrate for at least 15 minutes before

starting with data acquisition unless otherwise specified. After the test, the results were compared with the set acceptance criteria.

2.2.1.1. Preparation of caffeine solutions

A stock solution of caffeine with a concentration of 1000 mg L^{-1} was prepared by weighing 10 mg of caffeine standard and dissolving it in 10 mL of water. This stock solution was used in the preparation of different concentrations (300, 220, 140, 75, 60, 40, 10 and 5 µg mL^{-1}) of caffeine standard in water. All the resulting solutions were weighed and their masses were recorded.

2.2.1.2. Performance verification of the pump

The performance verification of the pump of the HPLC was done through the determination of flow rate accuracy, flow rate precision, and gradient accuracy.

2.2.1.2.1. Determination of flow rate accuracy

The flow rate accuracy was determined by measuring the time elapsed to fill a 10 mL flask with water flowing from a solvent channel at a rate of 1 mL/min. The flow rate was calculated using the formula below:

$$\text{flow rate}\left(\frac{\text{mL}}{\text{min}}\right) = \frac{\text{volume of flask (mL)}}{\text{time to fill (min)}} \qquad \text{Equation 2.1}$$

The measurement was done in triplicate and then the average flow rate was compared with the set flow rate.

2.2.1.2.2. Determination of flow rate precision

The mobile phase (85:15 (v/v) water:acetonitrile) was set at a flow rate of 1 mL/min. The acquisition time was 6 minutes and the response of the UV detector was monitored at 272 nm. The flow rate precision was determined by repeated injections (10 times) of 5 µL of a 140 µg mL^{-1} caffeine standard solution. The retention time of caffeine was obtained after each injection. The %RSD of the retention time was used to evaluate the flow rate precision.

2.2.1.2.3. Determination of gradient accuracy

This determination was necessary to be performed for all the channels of the pump, using two channels for each determination. One channel was filled with mobile phase A (99.5:0.5 (v/v) methanol:acetone) while another channel was filled with mobile phase B (methanol). The flow rate was set to 1 mL/min and UV detection at 265 nm. The program for gradient accuracy testing is shown in Table 2.1. By performing a blank run, a chromatogram was obtained showing the absorbance change (expressed as height) as gradient changes from 100%B to 100%A and then back to 100%B. The gradient accuracy was calculated from the relative heights (expressed as %Height ratio) of %A (in each step gradient) to 100%A. The calculated %Height ratio was compared with the set value for %A.

$$\%\text{Height ratio} = \frac{\text{Height of \%A}}{\text{Height of 100\%A}} \qquad \text{Equation 2.2}$$

Table 2.1. Program for gradient accuracy testing.

Time (min)	% Mobile phase A	% Mobile phase B
0	0	100
3	0	100
6	100	0
9	100	0
9.2	80	20
12	80	20
12.2	60	40
15	60	40
15.2	40	60
18	60	40
18.2	80	20
21	80	20
21.2	100	0
25	100	0

2.2.1.3. Performance verification of the autosampler

The performance of the autosampler was verified by determination of injection volume precision, injection volume linearity and carryover.

2.2.1.3.1. Determination of injection volume precision

The mobile phase (85:15 (v/v) water:acetonitrile) was set at a flow rate of 1 mL/min. The acquisition time was 6 minutes and the response of the UV detector was monitored at 272 nm. The injection volume precision was determined by repeated injections (10 times) of 5 µL of a 140 µg mL^{-1} caffeine standard. The peak area of caffeine was obtained after each injection. The %RSD of peak area was used to evaluate the flow rate precision.

2.2.1.3.2. Determination of injection volume linearity

The mobile phase (85:15 (v/v) water:acetonitrile) was set at a flow rate of 1 mL/min. The acquisition time was 6 minutes and the response of the UV detector was monitored at 272 nm. The injection volume linearity was determined by injecting 5, 10, 20, 40 and 80 µL of a 10 µg mL^{-1} caffeine standard. The peak area of caffeine was obtained after each injection. A linearity plot (peak area vs. injection volume) was constructed and the linear regression coefficient (r) was obtained. The ratio between the peak area and the volume injected was calculated and the %RSD of the peak area/injection volume was also determined.

2.2.1.3.3. Determination of carryover

The mobile phase (85:15 (v/v) water:acetonitrile) was set at a flow rate of 1 mL/min. The acquisition time was 6 minutes and the response of the UV detector was monitored at 272 nm. The carryover was determined by injecting 5 µL of a 75 µg mL^{-1} caffeine standard. Immediately after running the standard, a 5 µL mobile phase was injected. The measurement was done in triplicate. The peak area of caffeine was determined in the standard and blank injections. The %carryover was calculated as follows:

$$\%\text{Carryover} = \frac{\text{peak area of caffeine in blank}}{\text{peak area of caffeine in standard}} \text{x} 100 \qquad \text{Equation 2.3}$$

2.2.1.4. Performance verification of the column oven

The column oven performance verification was done by determining the column oven accuracy, column oven temperature precision and column oven temperature stability. Instead of the column, an LC zero dead volume union was used to connect the column inlet and outlet tubings. The flow rate for water was set at 0.1 mL/min. A calibrated digital thermal probe (Testo 945) was used to measure the temperature inside the column oven. The measured temperatures were corrected using the correction factors cited in the thermometer´s calibration certificate.

2.2.1.4.1. Determination of column oven temperature accuracy

The column temperature was set to 20ºC. After the set temperature was reached, the temperature was recorded every three minutes. Three temperature readings were obtained. The difference between the corrected temperature and the set temperature was calculated. The same procedure was done at the set temperatures of 40ºC and 60ºC.

2.2.1.4.2. Determination of column oven temperature precision

The column temperature was set to 40ºC. After the set temperature was reached, the temperature was recorded. The temperature was decreased by setting to 35 ºC. The temperature was then again set to 40ºC and the temperature was recorded after the set temperature was reached. This procedure was done once more to obtain triplicate readings. The maximum difference between the 3 temperature readings was calculated.

2.2.1.4.3. Determination of column oven temperature stability

The column temperature was set to 40ºC. After the set temperature was reached, temperature measurement was started. Temperature readings were obtained every 4 minutes and for a period of 1 hour. A temperature stability plot was made between the temperature readings and time.

2.2.1.5. Performance verification of the detector

The performance verification of the detector was conducted by determining the linearity of detector response and the noise and drift.

2.2.1.5.1. Determination of the linearity of detector response

The mobile phase (85:15 (v/v) water:acetonitrile) was set at a flow rate of 1 mL/min. The acquisition time was 6 minutes and the response of the UV detector was monitored at 272 nm. The linearity of detector response was determined by injecting 5 µL each of 10, 60, 140, 220 and 300 µg mL^{-1} caffeine standards. The peak area of caffeine was obtained after each injection. A linearity plot (peak area vs. injection concentration) was constructed and the linear regression coefficient was obtained. The ratio between the peak area and the concentration was calculated and the %RSD of the peak area/concentration was also determined.

2.2.1.5.2. Determination of noise and drift

This determination was necessary to be performed for all the channels of the pump, using two channels for each determination. To determine the noise and drift, one channel was filled with water and the other channel was filled with methanol. The flow rate of the mobile phase (50:50 (v/v) methanol:water) was set to 1 mL/min. The UV detector was turned on and the system was stabilized for at least one hour before starting the data acquisition. The acquisition time was 20 min., and the response of the UV detector was monitored at 254 nm. A blank injection was performed in order to obtain the baseline plot. The baseline plot was divided into 20 segments and the noise and drift was calculated in each segment. The noise and drift was calculated using the Chromeleon software. The noise corresponds to the distance between two parallel lines through the measured minimum and maximum values and the regression line. The drift was estimated as the slope of the regression line.

2.2.2. Column Performance verification

The performance of the Ascentis® Express RP-Amide column (10cm x 2.1 mm, 2.7 µm, Supelco) was evaluated using two HPLC instruments: Accela HPLC-UV and Dionex-HPLC UV/Vis. The procedure of the test is described in the document entitled *Instructions For Verification Of Column Performance* (Ascentis Express® RP-amide, 10 cm x 2.1 mm x 2.7 µm, Supelco) (SOP/CECEM/EQP/07/01).

2.2.2.1. Preparation of test solution

The stock solutions of uracil (1mg mL^{-1}), acetophenone (1mg mL^{-1}) and toluene (10 mg mL^{-1}) were prepared as follows: weighing 5 mg of uracil and dissolving in 5 mL of 50:50 (v/v) acetonitrile:water solution; taking 5µL of acetophenone and mixing with 5 mL of 50:50 (v:v) acetonitrile:water solution; taking 58 µL of toluene and mix with 5 mL of 50:50 (v:v) acetonitrile:water solution. The test solution was prepared by taking aliquots of 20 µL of 1 mg mL^{-1}uracil, 30 µL of 1mg $mL^{-1}$of acetophenone, 240 µL of 10 mg mL^{-1} toluene and mixing with 710 µL of 50:50 (v:v) acetonitrile:water in a glass vial.

2.2.2.2. HPLC run

These were the chromatographic conditions used: mobile phase: 50:50 acetonitrile:water; flow rate: 0.5 mL/min; injection volume: 1 µL; acquisition time: 4 min; temperature: 25°C; UV detection: 254 nm.

The HPLC column was installed in the HPLC instrument and was allowed to equilibrate with the mobile phase for at least 15 minutes. The test compound was injected and after the chromatographic run the retention times of the eluted peaks were compared with the retention times indicated in the test chromatogram from the vendor. The number of plates (N), tailing factor (T_f) and capacity factor (k´) for the last peak (toluene) were calculated using Equations 2.4, 2.5 and 2.6, respectively. The calculated values were compared with the vendor´s specifications.

N was calculated from the following equation:

$$N = 5.545\left(\frac{t_R}{w_h}\right)^2 \qquad \text{Equation 2.4}$$

where t_R stands for the retention time and $w_{0.5}$ for the peak width at half height

The tailing factor (T_f) was calculated as:

$$T_f = \frac{W_{0.05}}{2f} \qquad \text{Equation 2.5}$$

where $W_{0.05}$ is the peak width at 5% height and f is the front half-width at 5% of the peak height.

The capacity factor or retention factor (k´) was calculated as

$$k' = \frac{t_R - t_0}{t_0}$$ Equation 2.6

where t_R stands for the retention time and t_0 for the retention time of the unretained compound or dead time.

2.2.3. Mass spectrometer performance verification

The procedure for this determination can be found on the document *Instructions for Performance Verification of the LCQ MS (Finnigan)* SOP/CECEM/EQP/05/01. The ion gauge pressure and the convectron gauge pressure were always checked before starting any MS analysis to ensure that the vacuum system is working properly. To demonstrate that the instrument´s major electronic systems were operating satisfactorily, the built-in option "Diagnostic Test" of the software was selected. With this test, the power supplies, API temperatures, lenses and RF were tested. The instrument displays a Pass/Fail result to indicate whether all parts are working properly or not.

2.2.3.1. Mass spectrometer calibration

The mass spectrometer was calibrated by following the procedure described in the document that already exists in the laboratory entitled *Instrucciones para la Calibracion y Tuning del Espectrómetro de Masas LCQ (Finnigan)* (PNT 035100 APR/103).

2.2.3.1.1. Preparation of the calibration solution

The calibration solution was prepared from the stock solutions of caffeine (1mg mL^{-1}), MRFA (5 nmol/µL) and Ultramark 1621 (0.1%). The caffeine stock solution was prepared by weighing 1 mg caffeine and dissolving it in 1 mL methanol; the MRFA stock solution by weighing 3.0 mg of L-methionyl-arginyl-phenylalanyl-alanine acetate·H_2O (MRFA) and dissolving in 1 mL of 50:50 (v/) methanol:water solution; and Ultramark 1621 solution by dissolving 10µL of Ultramark 1621 in 10 mL of acetonitrile. A 5 mL calibration solution was prepared by pipetting the following into a clean, dry vial: 100 µL caffeine stock solution, 5 µL MRFA stock solution, 2.5 mL

Ultramark 1621 stock solution, 50 µL glacial acetic acid, and 2.34 mL 50:50 (v/v) methanol: water solution.

2.2.3.1.2. Instrument Setup

The fused silica capillary used for calibration was connected in the ESI probe before the probe assembly was installed in the detector. The ESI probe was configured to work at low flow rate infusion. The syringe was filled with the calibration solution and was connected directly to the grounded fitting of the probe assembly.

2.2.3.1.2. Calibration

The instrument was set to the ESI positive mode and the calibration solution was infused at a flow rate of 3µL/min. The ESI source parameters were set to the following values: Sheath gas flow rate: 40; Aux. Gas flow rate: 0; Spray voltage: 4.00; Capillary temperature: 275; Capillary voltage: 3.00; Tube lens offset: 30.00. The Define scan parameters used were: Scan mode: MS; Scan type: Full; MS^n power: 1; Number of Microscans: 2; Maximum inject time: 200; Input Method: From Mass 150 to 2000; Source Fragmentation: Off.

The ESI operation was first tested by observing the singly-charged positive ions for caffeine, MRFA, and Ultramark 1621. Before calibration was done, the instrument response was optimized by automatic tuning (via the instrument´s Tune program) using the caffeine peak of m/z 195. Afterwards, the automatic calibration was begun by selecting the instrument´s Calibrate option. Once the calibration has finished, a calibration report was displayed showing the success/fail result of the calibration. After calibration, the fused silica capillary was removed from the ESI probe and was replaced with a new capillary. The detector was flushed with acetonitrile for cleaning.

2.2.4. LC-MS performance verification

The proper functioning of the LC-MS was verified by using a method that was developed in the CECEM laboratory. The method involves the LC-MS/MS analysis of naphthylacetics (1-naphthoxyacetic acid, 2-naphthoxyacetic acid and 1-

naphthylacetamide). For the purpose of this investigation, it was only necessary to determine the possible conditions in which this method can be adopted in the Finnigan LCQ MS and determine some quality parameters such as limit of detection, limit of quantification, linearity and precision, which can serve as a reference for verifying the performance of the whole LC-MS system in the future. The procedure for the LC-MS verification can be found in the document entitled *Performance Verification of an LC-MS System* (SOP/CECEM/EQP/08/01) found in the Appendix.

2.2.4.1. Tuning with the analytes

Before starting with any LC-MS determination, the response on the MS detector has to be optimized by tuning the tube lens with 1-naphthylacetamide (5 µg mL^{-1}) and 1-naphthoxyacetic acid (5 µg mL^{-1}) using a 50:50 (v/v) methanol:2mM acetic acid solution as mobile phase. The standard solution of the analyte was introduced by infusion using the syringe pump. The scan parameters used were as follows: Scan mode: MS; Scan type: Full; MS^n power: 1; Number of Microscans: 3; Maximum inject time: 100; Input Method: From Mass 50 to 300; Source Fragmentation: Off. The tuning process was done by using the Tune program of the instrument software. Semi-automatic tune was done for the other MS parameters (capillary voltage and tube lens offset voltage).

2.2.4.2. Establishment of the chromatographic and MS detection conditions

2.2.4.2.1. Liquid chromatographic conditions

From the original method, the same gradient elution program was employed but the flow rate was decreased to 300 µL/min due to the limitations imposed by the HPLC-MS instrument. The mobile phase A (2mM acetic acid) was prepared by adding 115 µL of glacial acetic acid to 1L of water. Mobile phase B is methanol and hence preparation was not necessary. The mobile phase composition is shown in Table 2.2. Finally, these were the conditions that were established to be adequate in performing the analysis:

Column: Ascentis Express RP-Amide, 10 cm x 2.1 mm, 2.7µm
Mobile Phase: 2 mM acetic Acid: Methanol
Flow rate: 0.300 mL/min
Column Temperature: 50°C
Injection volume: 5 µL

Table 2.2. Gradient elution program.

Time, min	%Acetic acid	%Methanol
0	70	30
2	70	30
4	55	45
5	55	45
8.4	30	70
11.8	30	70
12.6	70	30
16.6	70	30

2.2.4.2.2. MS conditions

The MS/MS detection settings were established in terms of the ESI source parameters, isolation width, normalized collision energy, activation Q and activation time. The optimum parameters were chosen such that the mass chromatogram shows the maximum product ion intensities for a selected precursor ion. Table 2.3 summarizes the optimal values of the MS parameters for this determination.

Table 2.3. MS detection parameters.

Parameters	1-Naphthylacetamide	1-Naphthoxyacetic acid	2-Naphthoxyacetic acid
ESI mode	positive	negative	negative
ESI Source parameters			
Sheath gas (arb)	70	53	53
Auxiliary gas (arb)	40	48	48
Spray voltage (kV)	4	4	4
Capillary Temperature (°C)	250	250	250
Capillary voltage (V)	9	-17	-17
Tube lens offset (V)	-15	15	15
Precursor (*m/z*)	186.1	201.1	201.1
Product ion for quantitation (*m/z*)	141	143	143
Product ion for confirmation (*m/z*)	169	157	157
Isolation width (*m/z*)	1.5	1.5	1.5
Normalized collision energy (%NCE)	25	29	28.5
Activation Q	0.40	0.40	0.40
Activation time (msec)	0.30	0.30	0.30

*arb = arbitrary units

2.2.4.3. Determination of quality parameters

The following quality parameters were evaluated for the determination of 1-naphthylacetamide, 1-naphthoxyacetic acid and 2-naphthoxyacetic acid by HPLC-MS/MS: limit of detection, limit of quantitation, linearity and precision (repeatability). These analytes were detected using the Finnigan LCQ MS analyzer equipped with an electrospray ion source operated in the positive mode (for 1-NAD detection) and negative mode (for 1-NOA and 2-NOA detection).

2.2.4.3.1. Preparation of standard solutions

A 1,000 µg mL^{-1} stock solution of each of the analytes was prepared from the following solid standards: 1-naphthoxyacetic acid, 98% purity; 2-naphthoxyacetic acid, 98% purity; 1-naphthylacetamide, 99% purity. A 5.00 mg solid standard was weighed and dissolved in 5 mL of methanol.

An intermediate standard (10 µg mL^{-1}) was made by pipetting 30 µL of 1,000 µg mL^{-1} 1-NOA, 30 µL of 1,000 µg mL^{-1} 2-NOA and 30 µL of 1,000 µg mL^{-1} 1-NAD and mixing with 3.910 mL of methanol. From this 10 ppm intermediate standard, working calibration standard solutions between 0.1 and 1 µg mL^{-1} was used for linearity, precision and recovery determinations. Another intermediate standard of 0.200 µg mL^{-1} concentration was prepared from 10 µg mL^{-1} standard. The 0.200 µg mL^{-1} standard was used for the preparation of standards (2.5 ng mL^{-1} to 50 ng mL^{-1}) used in LOD determination. The mass of the aliquot taken and mass of the final solution were recorded and used for the calculation of the final concentration.

2.2.4.3.2. Identification of the analytes

The analytes were identified by injecting individual standard solutions of each analyte at 0.5 µg mL^{-1}. From the resulting chromatogram, the retention times were noted and the mass tandem spectrum was examined for confirming the presence of the precursor and product ions.

2.2.4.3.3. Determination of limit of detection (LOD) and limit of quantitation (LOQ*)*

The instrument LOD was determined by preparing dilute standard solutions from a 0.200 µg mL^{-1} standard containing a mixture of 1-naphthoxyacetic acid, 2-naphthoxyacetic acid and 1-naphthylacetamide. The LOD and LOQ were estimated based on the signal-to-noise ratio (S/N) measurement. The dilute standard solutions were subjected into the LC-MS run, and the chromatogram obtained was inspected for the S/N ratio. LOD was estimated as the standard concentration (ng mL^{-1}) which gave a S/N ratio around 3. Finally, the LOD was expressed as the amount (ng) of analyte injected by the expression:

LOD (ng) = Concentration (ng/µL) * volume (µL) injected Equation 2.7

The LOQ was estimated from the LOD data. LOQ (S/N ≈ 10) was derived from the expression:

LOQ = LOD * 3.3 Equation 2.8

2.2.4.3.4. Determination of linearity

The linearity was determined by preparing standard solutions containing the three analytes from a concentration similar to the LOQ until around 1 µg mL^{-1}. The standards were injected starting from the lowest to the highest concentration. At the end, the peak areas were determined by manual integration on the Xcalibur software. A plot of peak area vs. concentration was made and the linear regression parameters were obtained. The linearity was evaluated in terms of the regression coefficient (r).

2.2.4.3.5. Determination of precision (repeatability) and relative error

The instrument precision was determined by performing 6 injections of a standard solution containing the analyte at a middle concentration level. The retention times and peak areas (manually integrated) were obtained from each run. The injection precision was evaluated in terms of the %RSD of the retention time and peak area. The precision was also evaluated in terms of the %RSD of the calculated concentration when the standard is quantified as unknown sample using the linear calibration curve.

Moreover, the error associated on the quantification was estimated by the %Relative error, which was calculated as follows:

$$\%\,Relative\,error = \frac{(concentration\,obtained\,from\,the\,calibration\,curve - theoretical\,concentration)}{theoretical\,concentration} x\,100 \qquad \text{Equation 2.9}$$

3. RESULTS AND DISCUSSION

Quality control is an important aspect of an analytical laboratory. Quality control measures must be designed and followed because they provide a mechanism in achieving reliable data. For a typical LC-MS determination, these are some of the measures necessary for obtaining quality results: (1) performance verification of HPLC, (2) performance verification of column, (3) performance verification of the MS system, (4) performance verification of the LC-MS system, and (d) documentation of the procedures necessary for carrying out these tasks.

After carrying out the different activities mentioned above, the corresponding documents were generated. The list of documents with the codification is shown in Table 3.1. All of the documents can be found in the Appendix section.

Table 3.1. Summary of the generated documents.

TITLE	CODE
Instructions for the Operation of Dionex HPLC System with UV-Vis Diode Array Detector	SOP/CECEM/EQP/01/01
Instructions for the Performance Verification of the Dionex HPLC System with UV-Vis Diode Array Detector	SOP/CECEM/EQP/02/01
Instructions for the Maintenance of the Dionex HPLC System with UV-Vis Diode Array Detector	SOP/CECEM/EQP/03/01
Instructions for the Operation of the LCQ MS (Finnigan) in ESI mode	SOP/CECEM/EQP/04/01
Instructions for Performance Verification of the LCQ MS (Finnigan)	SOP/CECEM/EQP/05/01
Instructions for the Maintenance of the LCQ MS (Finnigan)	SOP/CECEM/EQP/06/01
Instructions for Verification of Column Performance (Ascentis Express® RP-amide, 10 cm x 2.1 mm x 2.7 µm, Supelco)	SOP/CECEM/EQP/07/01
Performance Verification of an LC-MS System	SOP/CECEM/EQP/08/01

3.1. Performance Verification of the Dionex HPLC-UV

The performance characteristics of the different HPLC modules were verified by determination of the different parameters listed in Table 3.2. In order to carry out these processes and set the acceptance criteria, the operational qualification (OQ) procedures performed by the instrument vendor were consulted along with the proposal from some existing guidelines. Some modifications were made in order to accommodate the current conditions and demands for a given parameter. Caffeine has a well-characterized UV

absorption profile and hence was used for most of the verification activities. The column temperature was set to 25°C except during verification of the column oven.

Table 3.2. HPLC performance verification parameters and acceptance criteria.

Module	Performance Attributes	Acceptance Criteria	Frequency
Pump	Flow rate accuracy	±2% of the set flow rate	6 months
	Flow rate precision	< 1% RSD	6 months
	Gradient accuracy	±1% of the step gradient composition	6 months
Injector	Injection precision	<1% RSD	6 months
	Injection volume linearity	r ≥ 0.999 %RSD (peak area/injection volume ratio) ≤5%	12 months
	Injection carryover	<1.5%	6 months
Column oven	Thermostatting accuracy	±3ºC maximum deviation from the set temperature	6 months
	Thermostatting precision	±0.5ºC maximum difference	6 months
	Temperature stability	±2ºC maximum difference	6 months
Detector	Linearity of detector response	r ≥ 0.999 %RSD (response ratio) ≤5%	12 months
	Noise and drift	Noise: 0.200 mAU Drift: 0.800 mAU/h	months

3.1.1. Verification of the pump

3.1.1.1. Determination of the flow rate accuracy

Pump is an essential component of an HPLC system that ensures an accurate and consistent flow of the mobile phase in order to have an efficient interaction between the stationary phase and the analyte. The flow rate of the mobile phase affects the time the analyte spends in the stationary phase and hence affects the time and degree of separation of the components in a given sample.

The flow rate accuracy was determined by setting the flow rate of water to 1 mL/min and measuring the time it took to fill a 10-mL volumetric flask. Three replicate analyses were done and the results are shown in Table 3.3. The average flow rate was 1.00 mL/min. Since the acceptance criteria set for the flow rate accuracy was 1.00 ± 0.02 mL/min, the results are acceptable.

Table 3. 3. Pump flow rate accuracy results.

Replicate	Time (min:sec:csec)	Flow rate (mL/min)
1	10:00:12	1.00
2	10:01:47	1.00
3	09:58:40	1.00
Average		**1.00**

3.1.1.2. Determination of the flow rate precision

The precision of the flow rate was determined by ten injections of 149 µg g^{-1} caffeine standard. The precision is expressed in terms of the % RSD of the retention times. The results are shown in Table 3.4. The calculated %RSD was 0.25%, so the system passed the set acceptance criteria (<1%RSD).

Table 3.4. Flow rate precision as determined by variability of the retention times of caffeine standard.

Replicate	Retention time (min)
1	3.410
2	3.427
3	3.432
4	3.434
5	3.437
6	3.429
7	3.429
8	3.422
9	3.417
10	3.419
Average	**3.426**
Standard deviation	**0.0084**
%RSD	**0.25**

3.1.1.3. Determination of the gradient accuracy

The Dionex HPLC-UV/Vis instrument is designed with left and right pumps, with each pump having three solvent channels. The gradient accuracy test is performed in all the solvent channels. As an example, the result of the gradient accuracy test for the two solvent channels of the left pump is mentioned here.

Gradient accuracy was evaluated by filling the solvent channel C with pure methanol and solvent channel A with 99.5: 0.05 (v/v) acetone:methanol solution. Decreasing the amount of acetone (a UV-active tracer) in the mobile phase leads to a decrease in UV absorption which enabled the accuracy of the gradient mixer to be

determined. In order to say that the pump is able to deliver the accurate solvent composition, there should be a proportional decrease in the peak height relative to the decrease in the acetone composition. The chromatogram for the gradient test is shown in Figure 3.1 with the corresponding gradient accuracy results on Table 3.5. All of the calculated % Height ratios are within the specified acceptance criteria (±1%).

Based on the results obtained here and from previous determinations such as flow rate accuracy and precision, it can be said that the pump is working properly under the set performance criteria.

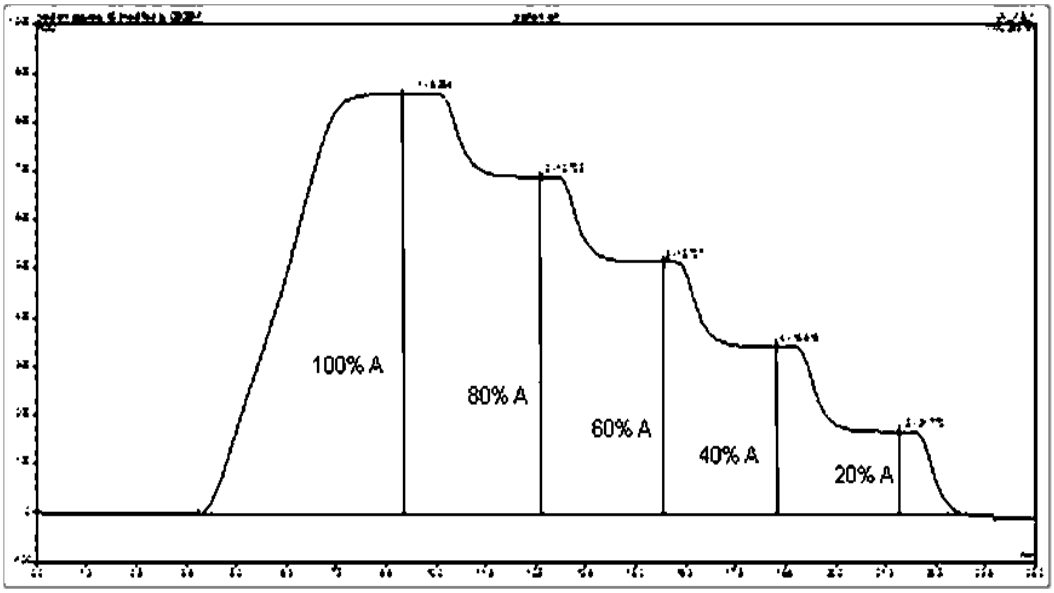

Figure 3.1. Gradient accuracy measurement.

Table 3.5. Measurement of the absorbance of acetone at varying mobile phase composition.

Step	Expected %A	Observed Height (mAU)	%Height Ratio	Deviation (%)	Result (Pass/Fail)
1	100	861.203	100.00	0.00	
2	80	691.216	80.26	0.26	Pass
3	60	516.472	59.97	0.03	Pass
4	40	342.908	39.82	0.18	Pass
5	20	169.151	19.64	0.36	Pass

3.1.2. Verification of the autosampler

3.1.2.1. Determination of the injection volume precision

The proper functioning of the autosampler is necessary since any error during autosampling will propagate through the separation, detection and quantitation and in the end will affect the final results. The ability of the injector to deliver the same amount of sample at the same speed and with the same mechanical motions during repeated injections is very crucial to the precision and accuracy of results obtained especially by external standard calibration. Many analysts prefer to use autosamplers than manual injection in order to obtain better repeatability (Hinshaw, 2000).

The injection repeatability was determined by performing 10 injections of a 140 µg g^{-1} caffeine solution. The precision was evaluated in terms of the %RSD of the peak areas. The results are shown in Table 3.6. The %RSD obtained was 0.16% which is less than the set acceptance criteria (%RSD <1%). Hence, it can be said that the autosampler is able to provide precise results.

Table 3.6. Injection volume precision as determined by variability of the peak areas of caffeine standard.

Replicate	Peak Area
1	28.4703
2	28.3785
3	28.3884
4	28.5241
5	28.3924
6	28.3834
7	28.2253
8	28.3795
9	28.4772
10	28.8514
Average	**28.4471**
Standard deviation	**0.16**
%RSD	**0.57**

3.1.2.2. Determination of the injection volume linearity

Linearity is important for methods requiring variable injection volumes. The uniformity of sample loop and the ability of the metering device to draw various amounts of sample in proper proportion will affect the linearity of the injection volume.

The linearity of the injection volume was demonstrated by injecting 5, 10, 20, 40 and 80 µL of a 16 µg g^{-1} caffeine solution. The peak area was determined after each injection and a calibration plot was prepared. The linear regression parameters were also obtained. The results are shown in Table 3.7 and the corresponding linearity plot is shown in Figure 3.2. The coefficient linear regression coefficient (r) is considered a suitable parameter for demonstrating the linearity over an appropriate linear range (Lam, 2004). Aside from this, the %RSD of the peak area/injection volume ratio was obtained as an aid for linearity evaluation. The regression coefficient was 0.9999 and %RSD (peak area/injection volume ratio) was 0.96%. With these results, the acceptance criteria ($r \geq 0.999$; ≤5% %RSD for peak area/injection volume ratio) were met.

Table 3.7. Linear response of the injection volume.

Injection volume, µL	Peak Area	Peak Area/Injection Volume Ratio
5	3.0095	0.6019
10	5.9291	0.5929
20	12.0447	0.6022
40	24.1573	0.6039
80	48.7095	0.6089

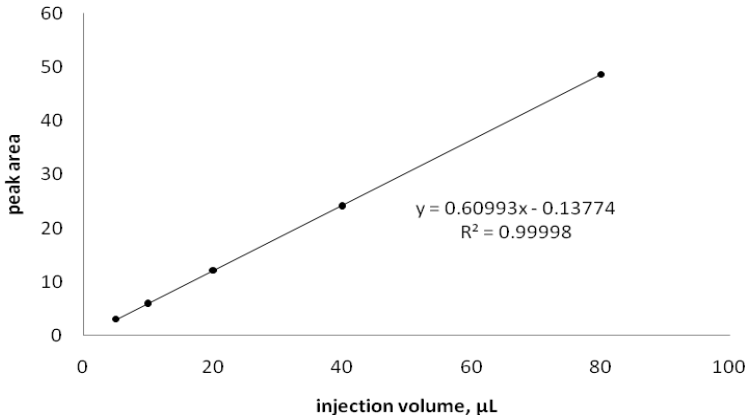

Figure 3.2. Linearity of the injection volume.

Table 3.8. Linear regression parameters for the injection volume.

Linear regression equation	0.60993x – 0.13774
R^2	0.9999
r	0.9999
%RSD (peak area/injection volume ratio)	0.96

3.1.2.3. Determination of carryover

The appearance of a peak in a blank injection after the injection of samples or standards with a high concentration (carryover) usually originates in the autosampler. Carryover can be a problem when analyzing a wide range of concentration as this could affect the accurate quantitation of the subsequent sample especially when working at low concentration levels (Dolan, 2001). The carryover was evaluated by performing a mobile phase injection after the injection of a 85 µg g^{-1} caffeine standard solution. The carryover was calculated from the peak area of caffeine in the standard and blank injections. An average carryover of 1.01% was obtained (See Table 3.8 for the results and Figure 3.3 for the sample chromatogram), which is within the set acceptance criteria of <1.5%. Carryover is method specific hence, for real applications, it may be necessary to determine the carryover using the specific method for a given analyte.

Table 3.9. Carryover determination.

Replicate		Retention time (min)	Peak area	%Carryover
1	Standard	3.480	18.5107	1.16
	Blank	3.524	0.2155	
2	Standard	3.495	18.2656	0.61
	Blank	3.506	0.1116	
3	Standard	3.507	18.6969	1.25
	Blank	3.567	0.2328	
			Average	**1.01**

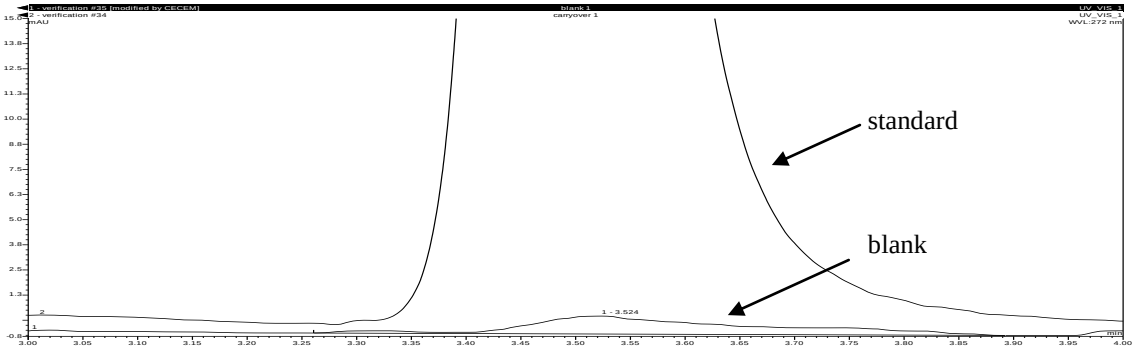

Figure 3.3. Sample chromatogram for the carryover determination.

Carryover usually results from one or two phenomena in the autosampler: (1) improper plumbing of the autosampler which creates reservoir in the system such that there is presence of small, unswept volumes after injection, and (2) sample adsorption to the tubing or other surfaces in the injection valve or autosampler. To avoid unswept

volumes in the system, the connections must be fitted properly and the drain lines must be clear so that the waste sample does not back up into the injection flow path. Sample adsorption in the autosampler can be avoided by a combination of increased flushing with the use of wash solvents sufficiently strong to displace the sample (Yuang, et.al.,1999).

3.1.3. Verification of the column oven

3.1.3.1. Determination of the column oven accuracy

Temperature is an important factor affecting column separations since it affects the retention time, capacity factor, resolution, selectivity and other variables. The determination of column oven temperature accuracy is important for comparability of results when transferring methods between systems (Bedsun and Rudd, 1999). The column oven accuracy was evaluated at three temperature settings: 20, 40 and 60°C. A calibrated digital thermal probe (Testo 945) was placed inside the column oven to measure the real temperature. The measured temperatures were corrected by using the correction factors provided in the calibration certificate of the thermometer. The corrected temperature readings were compared with the set temperature. The acceptance limit was set at ±3°C. The results are acceptable as shown in Table 3.10.

Table 3.10. Column oven accuracy results.

Set Temperature (°C)	Measured Temperature (°C)		Corrected Temperature (°C)	Deviation (°C)	Result (PASS/FAIL)
20	Reading 1	21.1	21.1	1.1	Pass
	Reading 2	21.1	21.1	1.1	Pass
	Reading 3	21.2	21.2	1.2	Pass
40	Reading 1	42.2	42.2	2.2	Pass
	Reading 2	42.2	42.2	2.2	Pass
	Reading 3	42.2	42.2	2.2	Pass
60	Reading 1	63.1	62.8	2.8	Pass
	Reading 2	63.2	62.9	2.9	Pass
	Reading 3	63.2	62.9	2.9	Pass
Maximum deviation (°C)				2.9	Pass

3.1.3.2. Determination of the column oven precision

The precision of the column oven is important for the repeatability of the retention times and peak area/peak height. The column oven precision was determined by setting and resetting the temperature at 40°C. The calibrated thermal probe was used to measure the real temperature inside the column oven. The results are shown in Table 3.11. The observed maximum difference between the three replicates was 0.5 ºC and this is still within the acceptance limit of ±0.5ºC.

Table 3.11. Column oven precision results.

Set Temperature (°C)	Measured Temperature (°C)	
40	Reading 1	41.7
	Reading 2	42.2
	Reading 3	42.0
Observed maximum difference (°C)		0.5

3.1.3.3. Determination of the column oven temperature stability

Using a column oven is a convenient way of controlling and maintaining a steady column temperature. The temperature inside the column oven should remain constant during the long period of analysis. The stability of the column oven temperature was determined over a 1 hour period by measuring the temperature inside the oven using the calibrated thermal probe. Within this 1 hour period, temperature readings were taken every 4 minutes. Table 3.11 shows the results obtained on the determination of the oven temperature stability. The corresponding stability plot is shown in Figure 3.4. The maximum difference between the temperature readings was determined to be 0.4°C. This is within the set acceptance criteria of ±2ºC maximum difference.

Table 3.12. Column oven temperature stability for a period of 1 hour.

Reading	Measured Temperature (°C)	Reading	Measured Temperature (°C)
1	42.1	9	41.9
2	41.8	10	41.8
3	41.8	11	41.7
4	41.9	12	41.8
5	41.8	13	41.8
6	41.8	14	41.8
7	41.8	15	41.8
8	41.8		

Minimum Temperature (°C)	41.7	Maximum Temperature (°C)	42.1
Maximum difference(°C)		0.4	

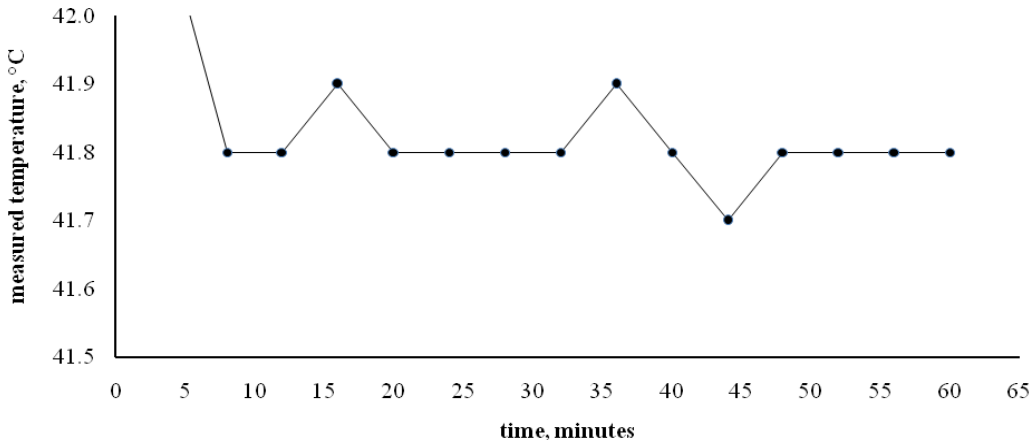

Figure 3.4. Column oven temperature stability over a 1 hour period.

3.1.4. Verification of the UV detector

3.1.4.1. Determination of the linearity of detector response

The detector must show a linear response with the change in the concentration of the analyte in order to obtain accurate results. The linearity of the detector response was evaluated by determining the peak area after the injection of different concentrations of caffeine standard solutions (16, 85, 149, 299 and 360 µg g^{-1}). A calibration plot of peak area vs. concentration was made and the linear regression parameters were calculated. The results are shown in Table 3.13 and the corresponding linearity plot is given in Figure 3.5. The regression coefficient was 0.99997 and %RSD (peak area/concentration ratio) was 1.01% (See Table 3.14). These results satisfy the acceptance criteria ($r \geq 0.999$; %RSD (peak area/concentration ratio) $\leq 5\%$).

Table 3.13. Determination of the linearity of UV-Vis detector response.

Concentration, µg g^{-1}	Peak Area	Peak Area/Concentration Ratio
16	3.0551	0.1905
85	16.0682	0.1886
149	28.1894	0.1897
299	55.6042	0.1860
360	67.1695	0.1867

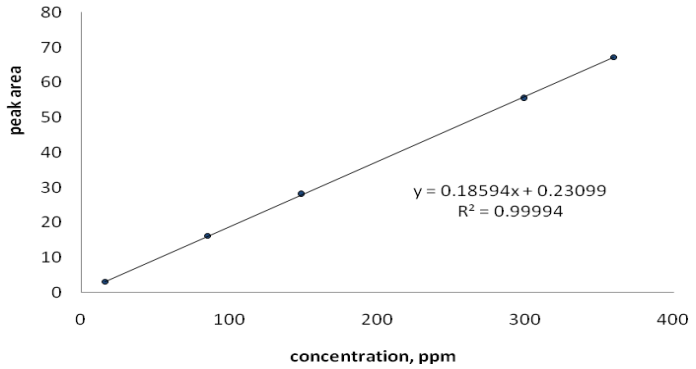

Figure 3.5. Linearity plot for the detector response.

Table 3.14. Linear regression parameters for the detector response.

Linear regression equation	0.18594x + 0.23099
R^2	0.99994
r	0.99997
%RSD (peak area/concentration ratio)	1.01

3.1.4.2. Determination of the noise and drift of the UV detector

The noise and drift of the UV detector is influenced by a lot of factors. Some of the causes of a noisy baseline include a low energy of the UV lamp, dirty flow cell, air bubble in the detector cell, pump not working properly and electrical signals from the environment. Baseline drift, on the other hand, may be due to electronics or from the strong pressure fluctuations of the pump which can be due to damaged pump valves and/or worn seals or a clogged pump inlet filter (Meyer, 2004). Baseline noise can be a limiting factor in the determination of trace components in a sample. Significant drift, on the other hand can affect proper peak integration. Changes in the baseline noise and drift over time should be monitored and evaluated.

The noise and drift were evaluated under dynamic conditions by measuring the detector signal over a 20-minute period as a steady flow of 50:50 (v/v) methanol:water passed through the system. Before the actual determination, the detector was turned on and the system was allowed to stabilize for at least an hour. The baseline plot obtained over the 20-minute period is shown in Figure 3.6. The baseline was divided into 20 segments (1 minute each segment) and in each segment, noise and drift were determined to yield the values shown in Table 3.15. The Chromeleon software was used in calculating these values. The average noise was 0.146 mAU and the drift was 0.212 mAU/h. Both of these results are acceptable according to the set acceptance criteria of 0.200 mAU for noise and 0.800 mAU/h for drift.

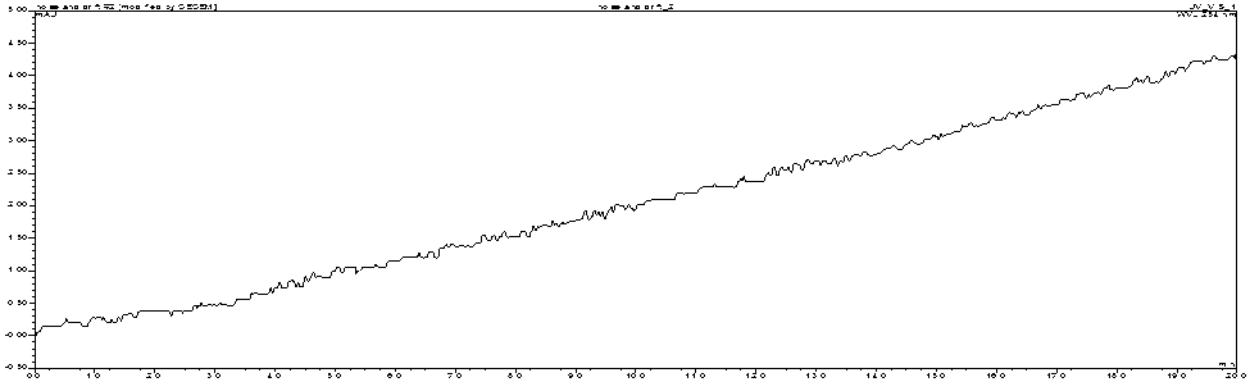

Figure 3.6. Baseline plot for the noise and drift determination.

The performance verification of the Dionex-UV was successfully done and the results are consistent with the set acceptance criteria. The standard operating procedures necessary to carry out these tasks were prepared as well as the forms where to put the acquisition data and results. These were all included in the Appendix.

Table 3.15. Noise and drift determination.

Segment	Noise (mAU)	Drift (mAU/h)
1	0.191	0.145
2	0.132	0.163
3	0.154	0.123
4	0.101	0.273
5	0.181	0.223
6	0.137	0.108
7	0.164	0.238
8	0.145	0.204
9	0.169	0.237
10	0.200	0.218
11	0.112	0.211
12	0.151	0.153
13	0.191	0.305
14	0.138	0.165
15	0.094	0.252
16	0.107	0.291
17	0.113	0.249
18	0.136	0.232
19	0.161	0.247
20	0.143	0.205
Average	**0.146**	**0.212**

3.2. Column Performance Verification

A newly bought column needs to be verified to assure that the specifications set by the vendor are met when the column is used in the laboratory. The performance of the Ascentis Express Amide column (10 cm x 2.1 mm, 2.7 µm, Supelco) was evaluated by following the conditions specified in the column test performed by the vendor. However, in the test provided, some important parameters were not defined such as the injection volume and the detection method used. When the performance test was adopted in the laboratory, it was necessary to set the given parameters and to specify the injection volume and method of detection used in order to carry out the test. The information obtained after column verification is needed for method development and for subsequent routine use.

The performance of the new column was evaluated by injecting a prepared test solution containing three of the four test compounds due to the unavailability of 4-Cl-nitrobenzene. The test was performed on two HPLC instruments found in the laboratory, the Accela and the Dionex-UV. The experimental results were compared

with the vendor´s specifications and an acceptance limit (for N) was set in order to have a basis for assessing the result as being acceptable or not.

The test chromatograms are shown in Figure 3.7 and the values for the different performance parameters are shown in Table 3.16. The experimentally obtained chromatogram was examined in terms of the retention times of the three compounds and the performance parameters for the more retained peak which was toluene. The retention time for uracil, which is an indicator of the column dead volume, differs in each instrument due to their configuration. No significant differences can be observed between the retention times obtained for acetophenone and toluene. The results for the tailing factor and capacity factor were acceptable.

Table 3.16. Vendor´s specification and column verification results.

Parameter	Vendor's specifications	Results	
		LC - Accela	LC-Dionex
Retention times (min)			
Uracil (t_0)	0.34	0.39	0.563
Acetophenone	0.74	0.81	0.928
Toluene	1.68	1.83	1.795
Performance results for toluene peak			
Plates (N)	15,000	12,198	2,721
USP Tailing factor	1.25	1.06	1.28
Capacity factor (k´)	3.92	3.69	2.19

The obtained values for the number of plates were below the vendor´s specifications. In the Accela instrument, the number of plates is about 81% of the minimum number of plates specified by the vendor, whereas, for the Dionex, the number of plates is about 18% of the minimum plates. The differences in the results can be attributed to the differences in instrument configuration between these systems.

In order to say that the column passed the performance test, the laboratory must set acceptance limits which are deemed practical for each instrument. It was suggested by Snyder, et.al. (1997) that if the determined N value for a new column is significantly lower, such that N < 80% of the claimed value, then it is necessary first to determine whether there is a possibility of instrumental problems otherwise the column should be returned to the vendor for replacement or refund. The experimentally determined N

values are normally lower than 100% of the specified N value and this can be attributed to the differences in the instrument used and the related extra-column problems in the system.

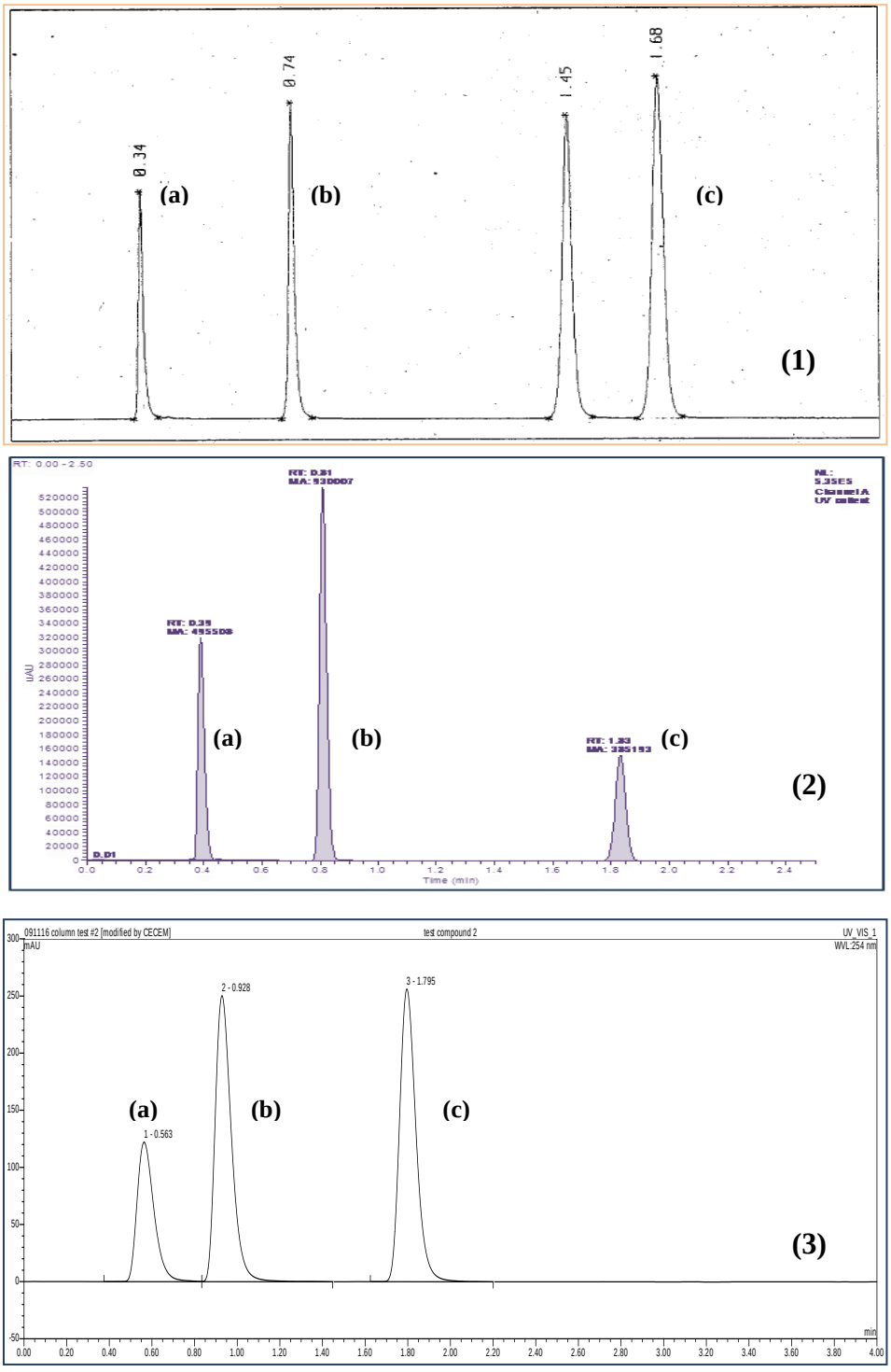

Figure 3.7. Test chromatograms from (1) vendor (2) Accela (3) Dionex-UV showing the elution of the test compounds: (a) uracil, (b) acetophenone and (c) toluene.

If we set that the N must be higher than 80% for new columns then the N result obtained using the Accela instrument are acceptable but not the results obtained with the Dionex system. The low N value for the Dionex instrument can be explained by the wider bandwidth observed for the toluene peak and the higher dead time obtained. The column is not just one factor that contributes to band broadening. Band broadening can also occur in the other parts of the HPLC system such as the injector/autosampler, connecting lines between the column and autosampler or detector, detector flow cell, etc. The existence of extra column band broadening leads to a lower N. The differences in the results obtained for these two instruments show that when we have to perform a column verification test, it is necessary that the instrument is well-plumbed so that there is minimum extra column band broadening that can be contributed to the results. Even though the results for the N value obtained using the Dionex instrument was far below the minimum, the results were accepted since the problem is not with the column but due to the nature of the instrument. The results were then filed for reference in future verifications and the document *Instructions for the Verification of Column Performance* (Ascentis Express® RP-Amide, 10 cm x 2.1 mm x 2.7 µm, Supelco) (SOP/CECEM/EQP/07/01) was prepared.

To ensure that the column is under quality control, column verification using the same test compounds must be performed periodically and a systematic record of the results must be maintained in the laboratory. In cases where there is already an existing standard HPLC method which makes use of this column, the user may determine the N value for their particular sample compound. The N value for the sample compound may be less than the optimum N value measured for a small neutral solute such as toluene. The differences in the determined N values are to be expected since N is dependent on several experimental factors.

A record of the column verification results will help the user to monitor the column performance and to anticipate when a column needs to be replaced. A column should be replaced whenever its performance falls down the expectations. For example, if the plate number decreases by 50% then a new column may be required. The type and number of samples injected contributes to column degradation hence it is suggested that a record is maintained regarding the use of the column. The best check in HPLC is

always the control chromatogram if there are deviations from previously defined results (Kromidas, 2000).

3.3. Mass spectrometer maintenance, performance verification, calibration and tuning

3.3.1. Mass spectrometer maintenance

Before starting with MS analysis, it is important that the instrument is properly maintained, calibrated and running at maximum sensitivity. Maintenance to be done on the mass spectrometer is to change the oil of the rotary-vane pump/forepump and clean the API source, the procedures of which can be found in the maintenance document *Instructions for the Maintenance of the LCQ MS (Finnigan)* (SOP/CECEM/EQP/06/01). As recommended by the manufacturer, the pump oil must be changed every 3 months or when the oil is cloudy or dark-colored as seen from the oil viewing port window. In practice, this frequency can be set to twice a year, depending on the current conditions. Maintenance of the API source involves replacement or cleaning of the sample tube, clearing the bore of heated capillary, cleaning the probe components and cleaning the API stack. Replacement of the sample tube is necessary if the sample tube is broken or obstructed; the frequency of cleaning (using methanol) of the probe components and the API stack depends on the instrument use and the nature of samples being analyzed.

The MS instrument is also maintained on a daily basis by purging the forepump and by flushing the sample transfer line, sample tube, spray shield, heated capillary and probe components by a 50:50 (v/v) methanol:water solution after the analysis. These are the responsibilities of the user of the instrument. The person responsible of the instrument and qualified laboratory personnel and serviceman perform the other maintenance activities on a regular basis.

3.3.2. Mass spectrometer performance verification and calibration

Prior to the calibration of the mass spectrometer, the vacuum levels have to be monitored and recorded: the ion gauge pressure should be around 1×10^{-5} Torr and the

convectron gauge pressure should be around 1 Torr (typically around 0.8 Torr). The convectron gauge pressure indicates the pressure in the capillary-skimmer region of the vacuum manifold and foreline, which connects the turbomolecular pump and the forepump. The ion gauge pressure, on the other hand, indicates the pressure in the analyzer region of the vacuum manifold. Vacuum pressures above these values indicate air leak in the system and hence it is necessary to check the tightness of fittings or flanges and it might be necessary to tighten them. Pressures below these values indicate that the heated capillary is partially or totally blocked.

To verify that the mass spectrometer is operating satisfactorily, the built-in option “diagnostic test” of the instrument has to be tested. This diagnostic test is used to check the major electronic circuits, voltage lenses, temperatures, gas flow rates, etc. within the instrument and indicate whether they pass or fail the tests. By performing this test, problems in the instrument can be located and corrected by replacing the faulty parts. Before running the LCQ diagnostic test, it is necessary to first tune the multipole frequency, ring electrode RF modulation and RF voltage frequency. The graphic results of the tuning processes which were performed are shown in Figure 3.8.

The tuning process for the multipole frequency was successful since the minimum frequency function was between 2400 and 2550 kHZ (See Figure 3.8a). Likewise, the RF modulation tuning (See Figure 3.8b) was also successful due to the following reasons: (a) the standing wave ratio switch line was at 10V, (b) the detected RF voltage was a straight line that begun at the origin and intersected the standing wave ratio switch line near the highest mass line, and (c) the RF modulation was a curved line that begun at the origin and intersected the highest mass line at around 4V. However, if the RF voltage frequency tuning is found to be lying outside the frequency window (as shown in Figure 3.8c), it is necessary to do manual adjustment of the RF voltage frequency by turning the tuning stud (which can be accessed by opening the front door of the MS detector) until the frequency cursor lies within the frequency window as shown in Figure 3.8d. This manual adjustment can only be done by the person responsible for the instrument.

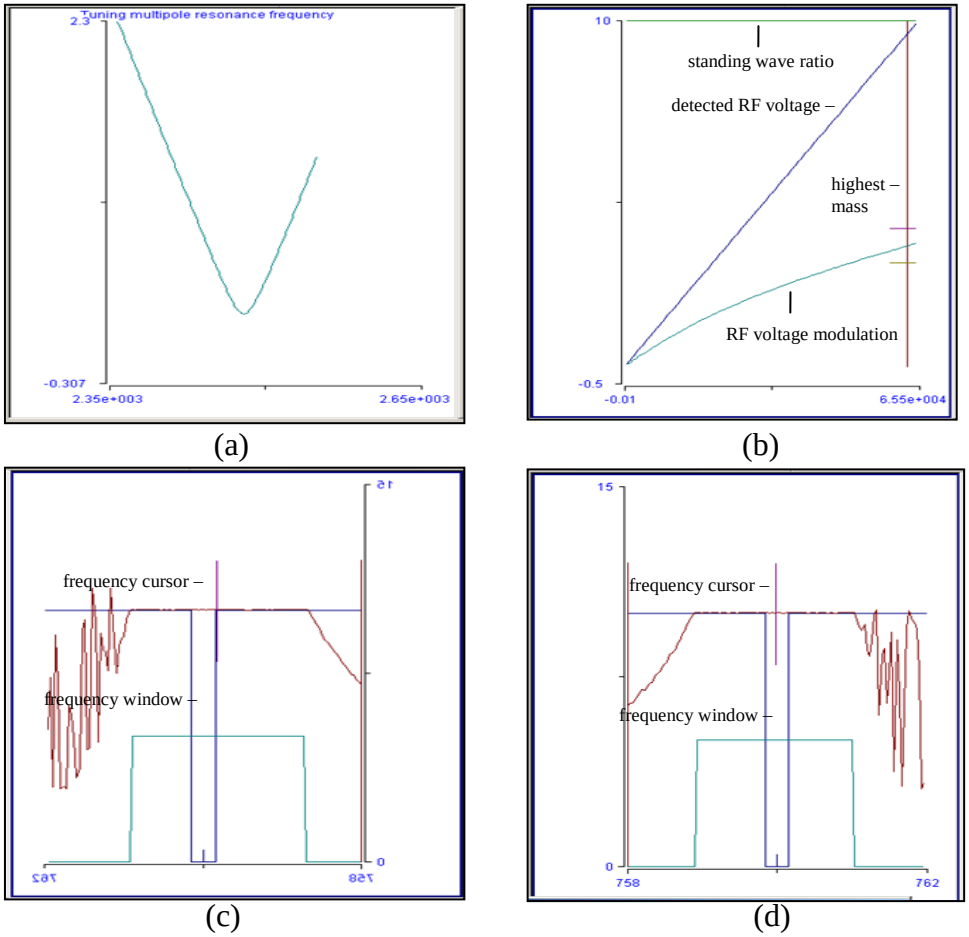

Figure 3.8. Graph view after the tuning process (a) multipole frequency, (b) RF modulation, (c) RF voltage frequency before manual adjustment and (d) RF voltage frequency after manual adjustment.

After the successful tuning process, the LCQ diagnostic was run by performing the test on the power supplies, API and temperatures, lenses and RF. The PASS results are shown in Figure 3.9, demonstrating the satisfactory performance of the electronic systems.

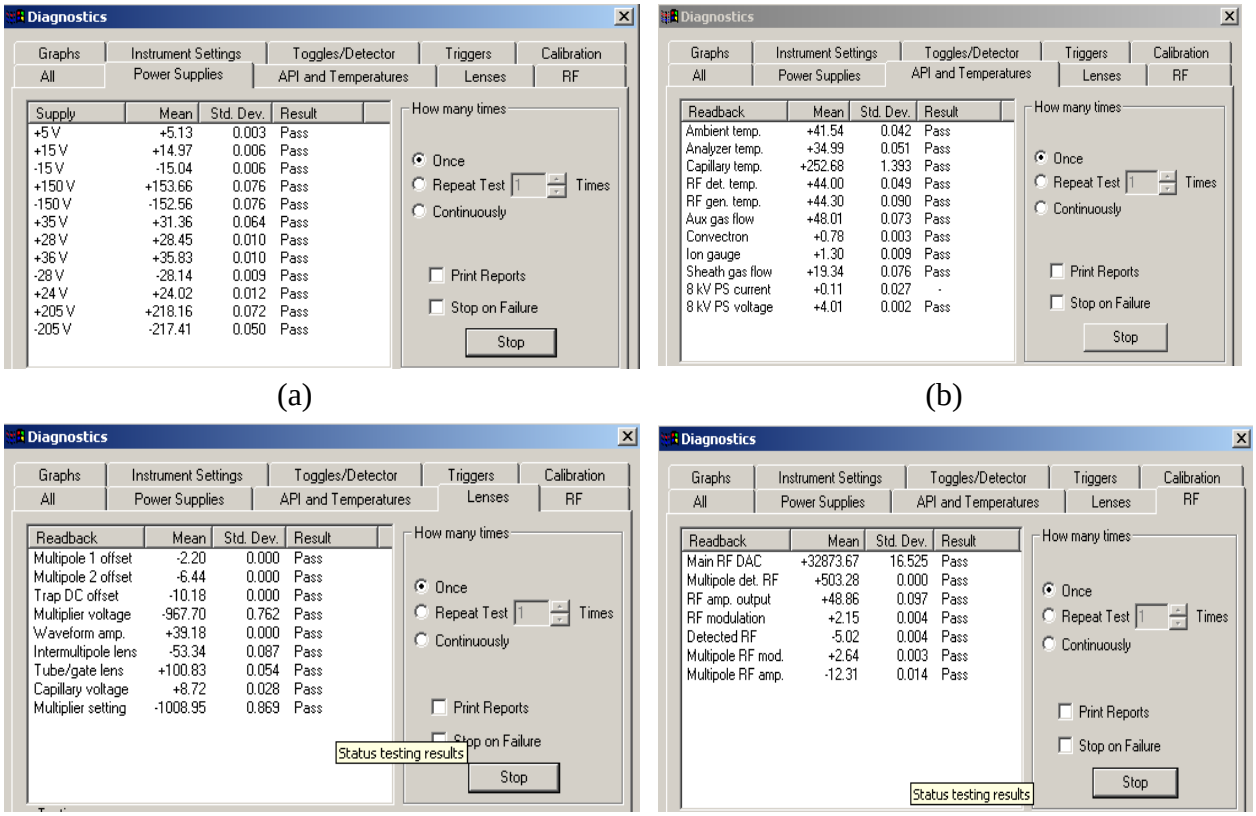

Figure 3.9. Diagnostic test results for (a) power supplies, (b) API and temperatures, (c) lenses and (d) RF.

3.3.3. Mass spectrometer calibration and tuning

The calibration of the LCQ mass spectrometer has to be performed by following the procedure described in the document entitled *Instrucciones para la Calibracion y Tuning del Espectrometro de Masas* LCQ (Finnigan) (PNT 035100 APR/103). This is a document that is implemented in the laboratory. The automatic calibration procedure was performed in the ESI positive mode by direct infusion using a calibration solution which contains the following compounds: caffeine, MRFA and Ultramark 1621. With this calibration mixture, different lenses are tuned and the *m/z* axis is calibrated over a *m/z* range from 50 to 2000 Th. The calibration frequency is recommended every three months (Garofolo, 2004) since the ion trap is a stable mass analyzer.

Before starting with the automatic calibration procedure, the ion source and the transmission has to be tuned with one of the calibrants (caffeine). This ensures that the ESI source spray is able to produce a stable spray of ions and an intense signal enough to calibrate the MS. Once the automatic tuning was started, the instrument performed all

the necessary processes and in the end, a calibration report is displayed and showed if the calibration is successful or not. A summary of the calibration report is shown in Table 3.17. The calibration report has to be printed and archived for reference. After calibration, it is also necessary to run again the diagnostic test in order to check for the proper operation of the mass spectrometer. Once the above mentioned tests are passed, it can be considered that the mass spectrometer is successfully calibrated and it is operating under control.

Table 3.17. Summary of calibration.

Item	**Result**
Multipole frequency calibration	SUCCESSFUL
Multiplier gain calibration	SUCCESSFUL
Normal scan calibration resolution	SUCCESSFUL
Normal scan mass calibration	SUCCESSFUL
AGC scan mass calibration	SUCCESSFUL
Zoom scan resolution calibration	SUCCESSFUL
Zoom scan mass calibration	SUCCESSFUL
Waveform/frequency calibration	SUCCESSFUL
Injection RF calibration	SUCCESSFUL

During the daily operations, it is not always necessary to repeat the full calibration procedure but instead, the mass spectrometer has to be tuned for the specific analytes to optimize the MS response. The signal is normally affected by the following parameters: heated capillary temperature, tube lens offset voltage, capillary voltage and sheath gas flow rate. The settings for these parameters are dependent on the solvent flow rate, the mobile phase composition and the analyte chemical characteristics hence fine tuning should be done at the beginning of the MS analysis. By performing the analysis in a properly calibrated instrument and by optimizing/tuning the MS with a standard solution containing the analyte, a significant improvement can be attained in terms of sensitivity. This is illustrated in Figure 3.10, wherein the calibration curves of 1-naphthylacetamide are compared before and after MS instrument calibration.

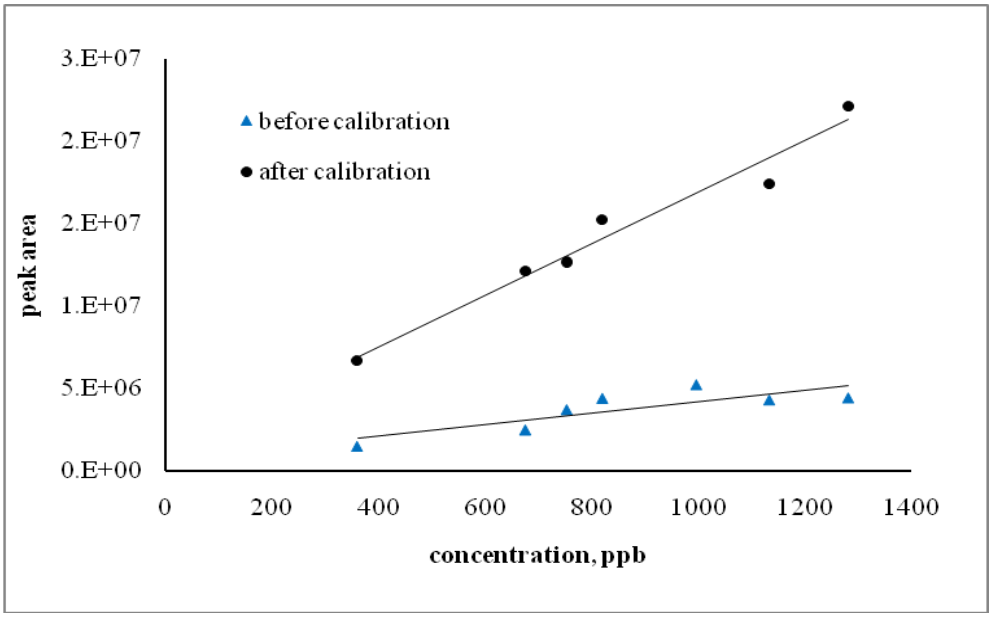

Figure 3.10. Effect of MS instrument calibration on the response for 1-naphthylacetamide.

3.4. LC-MS/MS performance verification using the analysis of naphthylacetics

During instrument performance verification, we are able to demonstrate that both instruments (LC and MS) are fit for a given purpose. However, when running a method using a hyphenated system (LC-MS) it is necessary to demonstrate its satisfactory performance. For this purpose, we use the analysis of naphthylacetics (1-naphthylacetamide, 1-naphthoxyacetic acid and 2-naphthoxyacetic acid) by LC-MS/MS. This is a previously established method in the CECEM laboratory. In order to demonstrate that the LC-MS/MS system is working properly, some quality parameters have to be determined such as limit of detection (LOD), limit of quantification (LOQ), linearity and precision (repeatability).

The method used is based on the determination of these naphthylacetics by LC-MS/MS. A gradient elution of methanol:water (2mM acetic acid) has been used for the separation of these compounds on an Ascentis® Express RP amide column (10 cm x 2.1 mm, 2.7 µm, Supelco) at a flow rate of 300 µL/min . Electrospray ion source operated in the positive mode for 1-naphthylacetamide (1-NAD) and negative mode for 1-naphthoxyacetic acid (1-NOA) and 2-naphthoxyacetic acid (2-NOA) was used to couple the LC system to the MS. The resulting chromatogram and spectrum is shown in Figure

3.11. The retention times for 1-NAD, 2-NOA and 1-NOA were 3.80, 8.31 and 9.07 min., respectively.

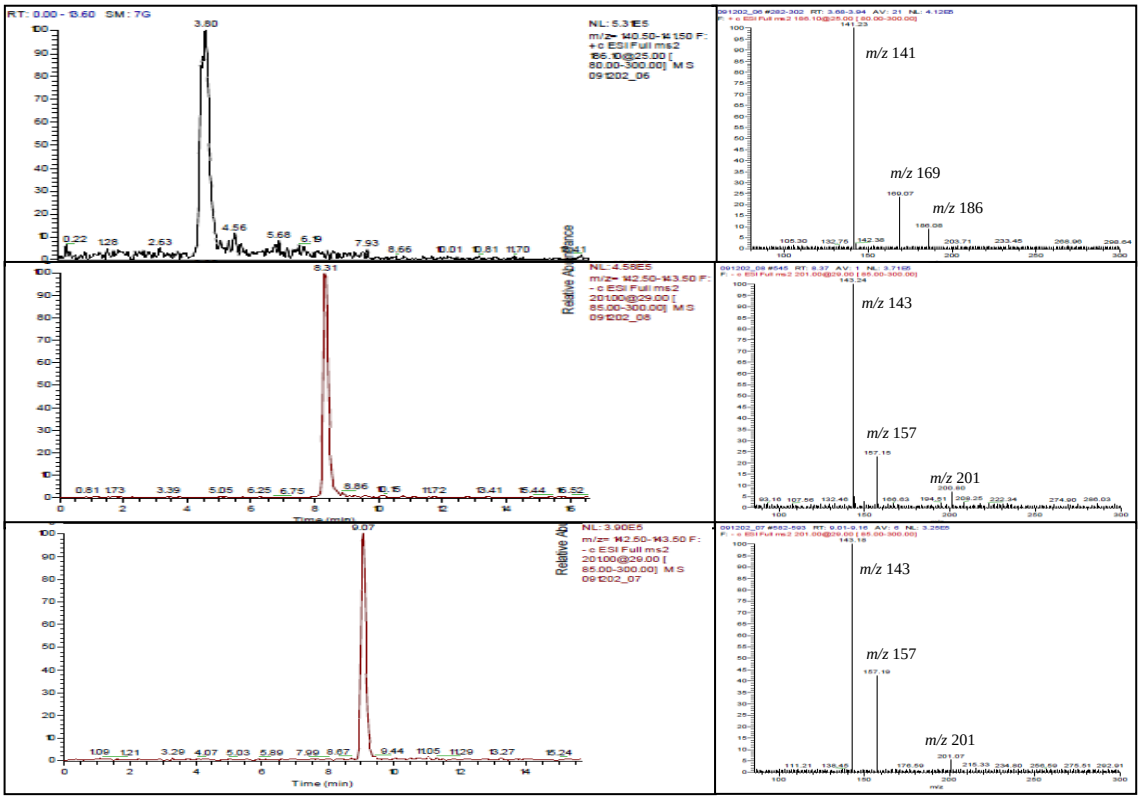

Figure 3.11. Individual chromatograms of (a) 1-naphthylacetamide, (b) 2-naphthoxyacetic acid, and (c) 1-naphthoxyacetic acid

3.4.1. Instrument LOD and LOQ determination

The instrument LOD and LOQ were determined on the basis of signal-to-noise ratio (S/N) measurement in order to check the sensitivity. Several dilute concentrations (4 ng g^{-1} up to 101 ng g^{-1}) of the standard solution containing the three compounds were prepared and subjected to the chromatographic run. The LOD was chosen as the analyte concentration giving S/N ≈ 3 at the quantitation *m/z* value. These concentration values correspond to 9.08 ng g^{-1}, 9.49 ng g^{-1} and 70.4 ng g^{-1} for 1-NOA, 2-NOA and 1-NAD respectively. The LOD values expressed in terms of the amount (ng) injected are shown in Table 3.18.

The LOQ values were likewise determined on the basis of the S/N ratio, such that the LOQ is equal to the amount of the analyte giving S/N≈ 10. These values were

estimated from the LOD data. The LOQ values for 1-NOA, 2-NOA and 1-NAD were 0.15, 0.16 and 1.16 ng, respectively.

Table 3.18. LOD and LOQ values for, 1-NOA, 2-NOA and 1-NAD.

Compound	LOD (ng)	LOQ (ng)
1-naphthoxyacetic acid	0.045	0.15
2-naphthoxyacetic acid	0.048	0.16
1-naphthylacetamide	0.352	1.16

3.4.2. Linearity

To check the linearity on the concentration, working range solution between 39 ng g^{-1} to 1.3 µg g^{-1} for 1-NOA, 37 ng g^{-1} to 1.3 µg g^{-1} for 2-NOA and 312 ng g^{-1} to 1 µg g^{-1} for 1-NAD have been prepared. The response (peak area) at each concentration level are shown in Table 3.19. The external standard calibration plots are shown in Figure 3.12. The linear regression equations were obtained by plotting the peak area against the concentration. The linear regression parameters are shown in Table 3.20. A good linearity can be observed for these compounds over the concentration ranges. A correlation coefficient (r) better than 0.99 is required for the acceptability of the linearity of the calibration plot.

Ideally, the linearity should be determined for several orders of magnitude until no linear response is observed. Nevertheless, to extend the calibration curve to very high concentration levels far from few ppm, possible contamination of the MS system can occur and cross contamination can be produced. For this reason, it is more practical to determine the linearity for a limited concentration range which is applicable to real samples. Then within this concentration range, the accuracy and precision of the method must be determined.

Table 3.19. Standard concentrations used for linearity determination.

2-NOA		1-NOA		1-NAD	
Conc., µg g^{-1}	Peak area	Conc., µg g^{-1}	Peak area	Conc., µg g^{-1}	Peak area
39.0	197707	37.3	182049	312.4	5709200
72.3	543274	69.2	392981	401.1	7204035
194.8	1291807	186.4	1140930	477.9	7936439
315.4	2509845	311.1	1746907	649.7	11790228
631.3	5521786	612.8	4295429	1038.8	16766901
887.8	7300464	847.3	6331372		
1,116.0	9218451	1,067.0	7240340		
1,313.0	11335562	1,282.0	9948370		

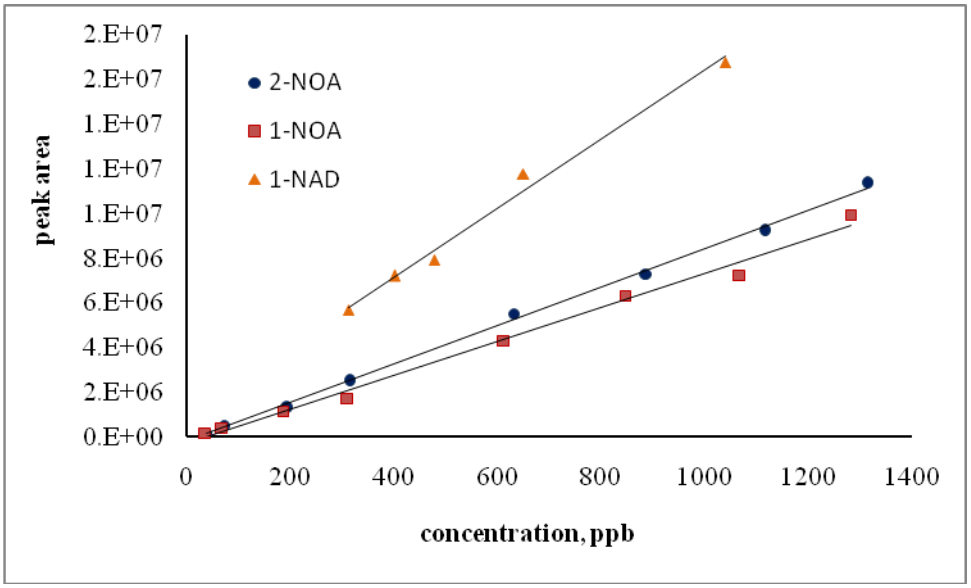

Figure 3.12. Calibration curves of 1-naphthylacetamide, 1-naphthoxyacetic acid and 2-naphthoxyacetic acid obtained by HPLC-MS/MS.

Table 3.20. Linear regression parameters for the different compounds.

Linear regression parameter	2-NOA	1-NOA	1-NAD
slope	8619	7606	19249
y-intercept	-183529	-286586	-2688369
R^2	0.99810	0.99146	0.99679
r	0.99905	0.99572	0.99839

3.4.3. Instrument Precision (Repeatability)

To check the precision of the response in LC-MS/MS, replicated injections (n=6) of a standard solution (315.4 ng g^{-1} of 2-NOA, 311.1 ng g^{-1} of 1-NOA and 477.9 ng g^{-1} of 1-NAD) have been performed. The precision was evaluated in terms of the %RSD of the retention time and peak area. As shown in Table 3.21, good precision can be observed relative to the retention times and peak areas of the three compounds. The %RSD for the retention times ranges from 0.35 to 1.77%. The %RSD of the peak areas ranges from 6.62 to 16.5%.

With the obtained data, it was also possible to determine the precision and the error associated with quantification. By making use of the external standard calibration curve, the concentration corresponding to the peak area was calculated for each replicate injection. This concentration was used for the %relative error calculations. The results for precision for the three compounds are shown in Table 3.21, Table 3.22 and Table 3.23.

Laboratories typically achieve a level of precision of <10% and in some instances the precision may be up to 20% especially near the limit of quantitation. The observed variability in the results is due to the contribution from several factors such as the instrument used, the analytical procedure, the noise level and peak integration. From the %RSD values, it can be said that required precision was attained.

Table 3.20. Repeatability expressed in terms of %RSD for the determination of 2-naphthoxyacetic acid.

Replicate	**t_R, min.**	**Peak area**	**Concentration, ppb**
1	8.38	2363108	295.5
2	8.32	2262004	283.7
3	8.31	2741692	339.4
4	8.38	2776389	343.4
5	8.35	2919322	360.0
6	8.35	2865389	353.7
Average	**8.35**	**2654651**	**329.3**
Std dev	**0.029**	**274236**	**31.8**
%RSD	**0.35**	**10.3**	**9.7**

Table 3.21. Repeatability expressed in terms of %RSD for the determination of 1-naphthoxyacetic acid.

Replicate	t_R, min.	Peak area	Concentration, ppb
1	9.05	1456225	229.1
2	8.98	1901898	287.7
3	8.98	1980046	298.0
4	9.04	1833270	278.7
5	8.98	2146205	319.9
6	8.98	2419074	355.7
Average	**9.00**	**1956120**	**294.9**
Std dev	**0.034**	**322167**	**42.4**
%RSD	**0.37**	**16.5**	**14.4**

Table 3.22. Repeatability expressed in terms of %RSD for the determination of 1-naphthylacetamide.

Replicate	t_R, min.	Peak area	Concentration, ppb
1	4.23	7027976	504.8
2	4.41	7560067	532.4
3	4.2	8275681	569.6
4	4.23	7818695	545.9
5	4.25	8483147	580.4
6	4.24	7812324	545.5
average	**4.26**	**7829648**	**546.4**
std dev	**0.0754**	**518031**	**26.9**
%RSD	**1.77**	**6.62**	**4.9**

The average %relative error obtained were 9.9%, 10.9% and 14.3% for 2-NOA, 1-NOA and 1-NAD, respectively. The acceptance criterion for the %relative error is method-dependent and should be appropriate for the intended use of the method. In this case, it can be set to not more than 15%. With this regard, the results for the relative error were acceptable. The deviation of the obtained concentration from the theoretical concentration can be partly attributed to the calibration curve used in estimating the concentration from the peak areas. Moreover, manual integration of the peaks can also affect the accuracy of quantification.

The results obtained fulfilled the set acceptance criteria and we conclude that the LC-MS/MS is running under control.

3.5. Documentation

Standard operating procedures (SOP) are essential part of quality system documentation. Although there were already existing procedures in the laboratory, most of these procedures were not formally stated and in order to achieve uniformity in carrying out specific tasks, SOP generation was necessary not only to provide evidence that the activities described above were carried out but also to serve as a reference for future use.

Standard operating procedures (SOP) were established for the following: (a) operation, maintenance and verification of the Dionex HPLC-UV, (b) verification of the performance of an RP-amide column, (c) operation, maintenance and verification of the Finnigan LCQ MS, and (d) verification of the performance of the LC-MS system through an in-house method. Whenever an SOP is to be written or revised, the author who will do the task must be involved in the process. It is for this reason that the author has familiarized with the method, instrument and laboratory facilities which were able provide very useful input for writing the necessary documentation.

The documents were written following a standard format in order to have uniformity. The format included the following features: (a) title page which indicates the university logo, document code, descriptive title, validity date, replacement information, names and signatures of responsible laboratory personnel, (b) table of contents (if applicable), (c) statement of objective, (d) statement of scope, (e) definitions, (f) related procedures, (g) responsibilities, (h) instructions and (i) bibliography.

The documents were codified in the following format: Type of Document/Laboratory Acronym/Specificity/Document Number/Revision Number. This is only a proposed codification since the documents are just in its initial phase to becoming official documents. The Type of Document can refer to a standard operating procedure (SOP) or other types of documents which in the future will be generated. The Laboratory Acronym is used to indicate that these documents belong to the CECEM laboratory where these documents will be used. The Specificity refers to whether it is for an equipment (EQP) or an analytical method (MET). The Document Number refers

to the number of the document generated in this study and the Revision Number refers to the version of the document which begins with 01 and continue as 02, 03, etc. An example of a part of the title page with the codification is shown in Figure 3.13. The form that is generated from a given SOP becomes a part of the document´s Annex. As an example, the title page generated from carrying out the performance verification of the HPLC is shown in Figure 3.14. It can be seen that the code in the performance verification form refers to the document code of the procedure (SOP) that was used to carry out the verification. All of the prepared documents are found in Appendix section as well as the external documents (test chromatogram result and calibration certificate for the digital thermometer used in performance verification of HPLC).

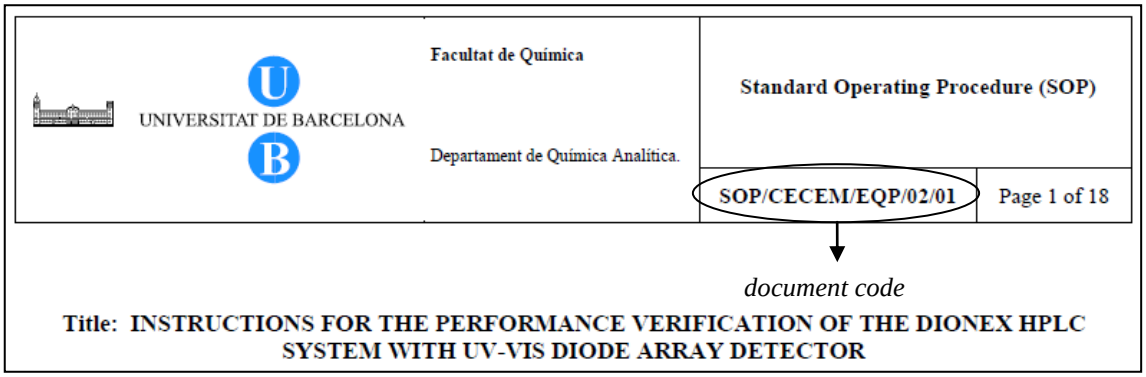

Figure 3.13. Part of the title page showing the document code for a standard operating procedure.

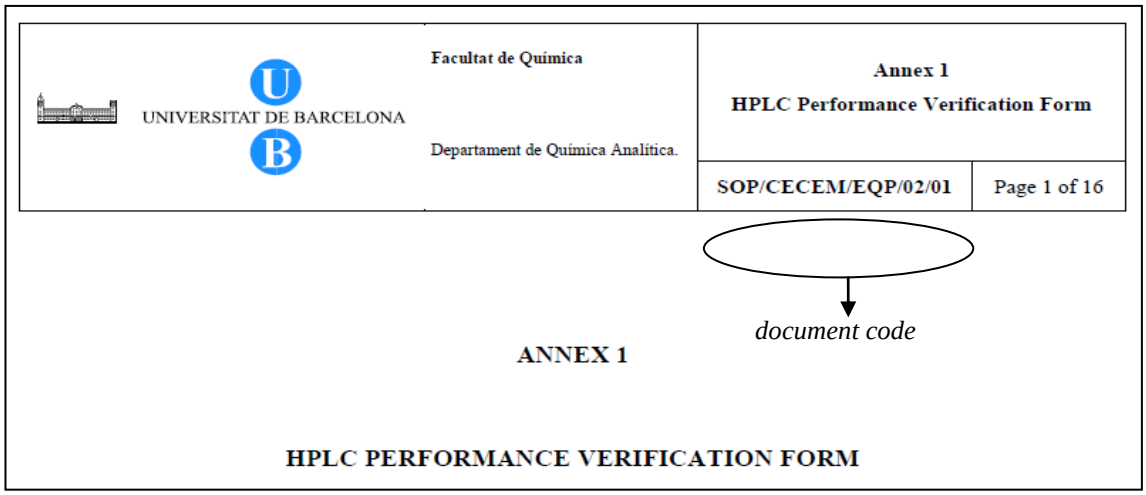

Figure 3.14. Part of the title page of a form showing the code in reference to the SOP where this form is used.

4. CONCLUSION

In this work, the standard procedures required for the control of an LC-MS system in a research laboratory have been developed. Since the LC-MS instrument is a coupled system composed of two main modules, a liquid chromatograph and a mass spectrometer, not only the control of the individual modules is necessary but the coupled system as well. For this reason, procedures for the LC, the MS and the LC-MS/MS system have been written.

The written documents have included standard procedures for the operation, verification and maintenance of the liquid chromatograph, the mass spectrometer and the coupled system (LC-MS). These documents have been applied in the research laboratory and they have guaranteed the right operation of the LC-MS system and have allowed the detection of failed components to be repaired or adjusted. These documents are part of the initial framework of the laboratory´s quality assurance system that in a near future would be implemented.

5. REFERENCES

Bansal, S. K., Layloff, T. Bush, E. D., Hamilton, M., Hankinson, E. A., Landy, S. J., Lowes, S., Nasr, M. M., St. Jean, P. A. and V. P. Shah. 2004. Qualification of Analytical Instruments for Use in the Pharmaceutical Industry: A Scientific Approach. AAPS PharmSciTech. 5(1):Article 22.

Bedson, P. and D. Rudd. 1999. The development and application of guidance on EQ of analytical instruments: High performance liquid chromatography. Accred Qual Assur 4:50-62.

Carson, P. A. and Dent, N. 2007. Good clinical, laboratory and manufacturing practices: Techniques for the QA professional. Cambridge: The Royal Society of Chemistry.

Currell, G. 2000. Analytical instrumentation performance characteristics and quality. England: John Wiley & Sons, Ltd.

Dass, C. 2007. Fundamentals of contemporary mass spectrometry. New Jersey: John Wiley & Sons, Inc.

Dionex Corporation. 2004. ASI-100 Autosampler Series Operating Instructions Version 2.00.

Dionex Corporation. 2004. P680 HPLC Pumps Operating Instruction, Revision 1.0.

Dionex Corporation. 2004. SOR-100 Solvent Racks Operating Instructions Revision 2.0.

Dionex Corporation. 2004. TC-100 Thermostated Column Compartment Operating Instructions Revision 3.00.

Dionex Corporation. 2005. Chromeleon Chromatography Management System Tutorial and User Manual Version 6.70.

Dionex Corporation. 2005. UVD 170U and UVD 340U UV/Vis Operating Instructions, Revision 1.1.

Dolan, J. W. 2001. Autosamplers, Part II-Problems and Solutions. LC-GC.19(2): 478-482.

Dong. M. W. 2006. Modern HPLC for Practicing Scientists. New Jersey: John Wiley and Sons, Inc.

EC. 2002. Commission Decision implementing Council Directive 96/23/ EC concerning the performance of analytical methods and the interpretation of results. Official Journal of the European Communities

Garofolo, F. 2004. In: Chan, C., Lam, H., Lee, Y.C., Zhang, X., eds. Analytical Method Validation and Instrument Performance Verification. Hoboken, New Jersey: John Wiley & Sons, Inc. pp.197-220

Hinshaw, J. V. 2000. Autosamplers-Symptoms and Solutions. LC-GC. 18(12):1234-1241.

International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. 2005. ICH Harmonised Tripartite Guideline. Validation of Analytical Procedures: Text and Methodology Q2(R1).

International Organization for Standardization (ISO). 2005. ISO 9000:2005(E) Quality Management Systems-Fundamentals and vocabulary. Geneva, Switzerland.

International Organization for Standardization (ISO). 2005. ISO/IEC 17025. General requirements for the competence of testing and calibration laboratories. Geneva, Switzerland.

International Organization for Standardization (ISO). 2007. ISO/IEC Guide 99. International vocabulary of metrology – Basic and general concepts and associated terms (VIM). First edition.

IUPAC. 1993. Nomenclature for chromatography. Pure & Appl. Chem. 65(4): 819-872.

Kenkel, J. 2000. A primer on quality in the analytical laboratory. Florida, USA: CRC Press LLC.

Kromidas, S. 2000 Practical Problem Solving in HPLC. Weinheim Germany: Wiley-VCH.

Krull, I. S., & Swartz, M. (1999). Mini-review analytical method development and validation for the academic researcher. Analytical Letters, 32 (6), 1067-1080.

Lam, H. 2004. Performance verification of HPLC. In: Chan, C., Lam, H., Lee, Y.C., Zhang, X., eds. Analytical Method Validation and Instrument Performance Verification. Hoboken, New Jersey: John Wiley & Sons, Inc. pp. 173-185.

Majors, R.E. 2003. The Cleaning and Regeneration of Reversed-phase HPLC Columns. LCGC Europe, 1-6.

Masson, P. 2007. Quality control techniques for routine analysis with liquid chromatography in laboratories. Journal of Chromatography A. 1158: 168-173.

Meyer, V. R. 2004. Practical high- performance liquid chromatography. 4th ed. Great Britain: John Wiley & Sons, Ltd.

Niessen, W. M.A. 2006. Liquid chromatography- Mass Spectrometry 3rd ed. NW, Suite: CRC Press.

Papadoyannis, I. N. and V. F. Samanidou. 2005. Validation of HPLC Instrumentation. Journal of Liquid Chromatography and Related Technologies. 27(5): 753 - 783

Prichard, E. & Barwick, V. 2007. Quality assurance in analytical chemistry. Teddington, UK: LGC Limited.

Ratliff, T. A. 2003. A manual of quality procedures and forms. Hoboken, New Jersey: John Wiley & Sons, Inc.

Simonet, B. M. 2005. Quality control in qualititative analysis. Trends in Analytical Chemistry, 24(6): 525-531.

Smith, P. 2007. Trends in Analytical Instrument Qualification. BioProcess International. 30-38.

Snyder, L.R., Kirkland, J.J. and J.L Giajch. 1997. Practical HPLC method development 2nd ed. New York: John Wiley & Sons, Inc.

Thermo Electron Corporation. 2003. Finnigan LCQ Hardware Manual 97000-97053 Revision B.

Wells, M., and M. Dantus. 2005. Jack Cazes ed. Validation of Chromatographic Methods In: Ewing´s Analytical Instrumentation Handbook. USA: Marcel Dekker.

Yuang. J.Y., Culley, T. and J.W. Dolan,.1999. Late elution and carryover peaks- A case study. LC-GC. 17(3): 230-234.

6. APPENDICES

Documents

Appendix Table 1. Document title and the proposed codification.

TITLE	CODE
Instructions for the Operation of Dionex HPLC System with UV-Vis Diode Array Detector	SOP/CECEM/EQP/01/01
Instructions for the Performance Verification of the Dionex HPLC System with UV-Vis Diode Array Detector	SOP/CECEM/EQP/02/01
Instructions for the Maintenance of the Dionex HPLC System with UV-Vis Diode Array Detector	SOP/CECEM/EQP/03/01
Instructions for the Operation of the LCQ MS (Finnigan) in ESI mode	SOP/CECEM/EQP/04/01
Instructions for Performance Verification of the LCQ MS (Finnigan)	SOP/CECEM/EQP/05/01
Instructions for the Maintenance of the LCQ MS (Finnigan)	SOP/CECEM/EQP/06/01
Instructions for Verification of Column Performance (Ascentis Express® RP-amide, 10 cm x 2.1 mm x 2.7 µm, Supelco)	SOP/CECEM/EQP/07/01
Performance Verification of an LC-MS System	SOP/CECEM/EQP/08/01