1.0 INTRODUCTION

1.1 Scope of the Method

Analytical method GRM051.08C is a revised method version of GRM051.08A for determination of acibenzolar-S-methyl and CGA210007 only (Figure 1) in water. The limit of quantification (LOQ) of the method has been established at 0.05 μg/L (or 0.05 ppb) for each of the analytes.

This method satisfies US EPA guideline EPA 850.6100 and EC Guidance Documents SANCO/3029/99 rev 4 and SANCO/825/00 rev 8.1.

1.2 Method Summary

Water samples are analyzed directly upon treatment with formic acid using high performance liquid chromatography with triple quadrupole mass spectrometric detection (LC-MS/MS).

The LOQ of the method is 0.05 μg/L (0.05 ppb).

2.0 MATERIALS AND APPARATUS

2.1 Apparatus

The recommended equipment and apparatus are listed in Appendix 1. Equipment with equivalent performance specifications may be substituted.

2.2 Reagents

All solvents and other reagents must be of high purity, e.g. glass distilled/HPLC grade solvents and analytical grade reagents. Particular care must be taken to avoid contamination of the reagents used. Reagents of comparable purity may be substituted as long as acceptable performance is demonstrated. A list of reagents used in this method along with details of preparation of solutions is included in Appendix 2.

2.3 Preparation of Analytical Standard Solutions

It is recommended that the following precautions should be taken when weighing the analytical materials.

1. Ensure good ventilation.
2. Wear gloves and laboratory coat.
3. Prevent inhalation and contact with mouth.
4. Wash any contaminated area immediately.
2.3.1 Stock Solutions

Prepare individual 50 - 200 µg/mL stock solutions for acibenzolar-S-methyl and CGA210007 by one of the following methods.

Weigh out accurately, using a five figure balance, sufficient acibenzolar-S-methyl and CGA210007 analytical standard into separate amber “Class A” volumetric flasks (50 mL size). Dilute to the mark with acetonitrile to give individual 50 - 200 µg/mL stock solutions of acibenzolar-S-methyl and CGA210007. Note that the amount weighed out must be corrected for its chemical purity.

Alternatively, the appropriate volume of acetonitrile to add to a known amount of standard material may be determined using the equation below. The standard concentration is corrected for its chemical purity.

\[ V = \frac{W \times P}{C} \times 1000 \]

\( P \) = Standard purity in decimal form (P(%)/100)

\( V \) = Volume of acetonitrile required

\( W \) = Weight, in mg, of the solid analytical standard

\( C \) = Desired concentration of the final solution, (µg/mL)

1000 = Unit conversion factor

In this case, the standard material is weighed directly into an appropriate storage vessel.

2.3.2 Fortification Solutions

Sample fortification solutions containing acibenzolar-S-methyl and CGA210007 should be prepared by mixing equal amount of aliquots from the stock solution and then serial dilution in acetonitrile. It is recommended that the following solutions are prepared: 10.0 µg/mL, 1.0 µg/mL and 0.1 µg/mL.

2.3.3 Preparation of Matrix-Matched Calibration Standards for LC-MS/MS

Matrix effects were observed from the water samples selected for the method ILV TK0211490, (Reference 5), for the primary transition or the confirmatory transition for acibenzolar-S-methyl and CGA210007. It is therefore recommended that matrix-matched calibration standards should normally be used for quantification of all analytes.

To prepare for example an LOQ equivalent 0.05 µg/L calibration standard in matrix water, accurately measure 50 mL of water (surface, ground or tap) into a 50 mL volumetric flask. Add 50 µL of concentrated formic acid to create acidic conditions < pH 5 resulting in a 0.1% formic acid solution. Then add 25 µL of 0.1 µg/mL mixed standard and cap the flask securely. Shake briefly to mix thoroughly.
A calibration curve using acibenzolar-S-methyl and CGA210007 standards over an appropriate concentration range should be prepared as described above, using the requisite volume of acibenzolar-S-methyl and CGA210007 standards in acidic matrix water (0.1% formic acid). A minimum of five levels of calibration standards should be used for calibration plot establishment. The following concentration levels of standards are prepared for acibenzolar-S-methyl and CGA210007 calibration plots: 0.01 pg/µL, 0.0125 pg/µL, 0.025 pg/µL, 0.05 pg/µL, 0.10 pg/µL, 0.30 pg/µL, 0.60 pg/µL and 1.0 pg/µL. Single point calibrations are not recommended for this method.

Typical chromatograms from LC-MS/MS analysis of the standard solutions are shown in Figures 4-19.

2.3.4 Standard Solution Storage and Expiration

All stock solutions should be stored in a refrigerator or freezer when not in use to prevent decomposition and/or concentration of the standard. Standard solutions should be allowed to equilibrate to room temperature prior to use.

Stability of working standards of acibenzolar-S-methyl and CGA210007 in acetonitrile has been demonstrated over a period of 53 days (Reference 2). Fresh standards should be prepared after this period unless additional data are generated to support a longer expiration date.

The matrix-matched calibration standard stability has been demonstrated for a period of 7-days (Refer to Section 8.6).

2.4 Safety Precautions and Hazards

The following information is included as an indication to the analyst of the nature and hazards of the reagents used in this procedure. If in any doubt, consult the appropriate MSDS or a monograph such as ‘Hazards in the Chemical Laboratory’, edited by S G Luxon, The Chemical Society, London (Reference 3).

**Solvent and Reagent hazards**

<table>
<thead>
<tr>
<th></th>
<th>Acetonitrile</th>
<th>Methanol</th>
<th>Formic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harmful Vapour</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Highly Flammable</td>
<td>✔</td>
<td>✔</td>
<td>✗</td>
</tr>
<tr>
<td>Harmful by Skin Absorption</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Irritant to respiratory system and eyes</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Causes severe burns</td>
<td>✗</td>
<td>✗</td>
<td>✔</td>
</tr>
<tr>
<td>OES Short Term (mg/m³)</td>
<td>105</td>
<td>310</td>
<td>19</td>
</tr>
<tr>
<td>OES Long Term (mg/m³)</td>
<td>70</td>
<td>260</td>
<td>9</td>
</tr>
</tbody>
</table>
Suitable personal protective equipment should be worn when handling chemicals and reagents. The appropriate MSDS should be consulted for each reagent and a local risk assessment should be carried out. In all cases avoid breathing vapour. Avoid contact with eyes and skin.

3.0 ANALYTICAL PROCEDURE

A summary of the method is included in flow-chart form in Appendix 4.

3.1 Sample Preparation

a) If water samples are received deep frozen they should be allowed to defrost completely at room temperature. Defrosted samples should be shaken thoroughly to ensure sample homogeneity prior to analysis. Any particulates may be removed by centrifugation at a speed which visibly separates the particulates from the water.

3.2 Sample Fortification and Analysis

Due to degradation of acibenzolar-S-methyl determined during the method development phase, samples are acidified to prepare an acidic condition at < pH 5 prior to fortification and analysis to increase acibenzolar-S-methyl stability. At least one untreated control and two fortified control samples should be analyzed with each sample set.

a). Fortification sample preparation: Aliquot 20 mL of water into a 50 mL polypropylene centrifuge tube. Add 20 µL of concentrated formic acid to yield a 0.1% formic acid solution in matrix water to create an acidic condition of <pH 5. Mix well by shaking. Fortify the recovery sample with the appropriate amount of each analyte using the mixed standard using no more than 0.1 mL. The sample is then shaken vigorously for 10 seconds to mix well.

b). Water sample preparation: Aliquot 20 mL of water into a 50 mL polypropylene centrifuge tube. Add 20 µL of concentrated formic acid to yield a 0.1% formic acid solution in matrix to create an acidic condition of < pH 5. The sample is then shaken vigorously for 10 seconds to mix well.

c) Samples are centrifuged or filtered through a 0.45 µm syringe filter if particles are visible before analysis.

d) An aliquot (approximately 1.5 mL) is transferred to an appropriate autosampler vial.

e) Final determination is performed by LC-MS/MS against matrix-matched calibration standards.
3.3 Experimental Precautions

a) Bottled HPLC grade ultra-pure water is used to prepare the LC mobile phase, which produces a lower background noise in the MS/MS chromatograms than water taken from a laboratory water purification system.

b) To prevent contamination of the instrument and to minimise possible carry-over issues, it is recommended that high level recoveries (>0.6 µg/L) and samples with expected residues greater than 0.6 µg/L should be diluted so that the final analyte concentration does not exceed 0.6 µg/L. It may also be useful to include blank injections of acetonitrile/ultra-pure water (50/50 v/v) after high level samples to clear any observed carry-over greater than 10% of the LOQ.

c) Additional needle and valve washes with an organic solvent such as acetonitrile and methanol may help to reduce any significant carry-over of acibenzolar-S-methyl in particular.

3.4 Time Required for Analysis

The methodology is normally performed with a batch of 20 samples. One person can complete the analysis of 20 samples in 0.5 day (8 hour working period).

3.5 Method Stopping Points

The analytical procedure can be stopped at various points for overnight and weekend breaks unless otherwise specified in the analytical procedure. Acceptable method recoveries will validate any work flow interruptions. Samples should be stored refrigerated in sealed containers where the analysis cannot be completed in a single day.

4.0 FINAL DETERMINATION

The method has been developed for use on an AB Sciex QTrap API5500. The following instrumentation and conditions have been found to be suitable for this analysis. Other instrumentation can also be used, though optimisation may be required to achieve the desired separation and sensitivity. The operating manuals for the instruments should always be consulted to ensure safe and optimum use.

4.1 Instrument Description

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pump</td>
<td>Agilent Technologies 1290 UPLC¹</td>
</tr>
<tr>
<td>Column Oven</td>
<td>Agilent Technologies 1290 Thermostatted Column Compartment</td>
</tr>
<tr>
<td>Detector</td>
<td>AB Sciex QTrap 5500 with Analyst™ software version 1.6.2</td>
</tr>
<tr>
<td>Autosampler</td>
<td>Agilent Technologies 1290 Infinity Autosampler</td>
</tr>
</tbody>
</table>
4.2 Chromatography Conditions for Acibenzolar-S-methyl and CGA210007

Column: Waters X-Select CSH C18 50 x 3 mm 2.5 μm

Column Oven Temperature: 40 °C

Injection volume: 100 μL

Stop Time: 6.0 mins

Mobile phase: Solvent A: 0.1% Formic Acid in HPLC Water
Solvent B: Methanol

1 The chromatographic system is a UPLC instrument: however, the method operates under HPLC conditions.

Mobile Phase Composition

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% Solvent 1</th>
<th>% Solvent 2</th>
<th>Flow, mL/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>80</td>
<td>20</td>
<td>0.5</td>
</tr>
<tr>
<td>2.0</td>
<td>10</td>
<td>90</td>
<td>0.5</td>
</tr>
<tr>
<td>4.5</td>
<td>10</td>
<td>90</td>
<td>0.5</td>
</tr>
<tr>
<td>4.6</td>
<td>80</td>
<td>20</td>
<td>0.5</td>
</tr>
<tr>
<td>6.0</td>
<td>80</td>
<td>20</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Under these conditions the retention time for CGA210007 is 2.74 mins and acibenzolar-S-methyl is 3.15 mins.
4.3 Mass Spectrometer Conditions for Acibenzolar-S-methyl

Interface : TurboIonSpray
Polarity : Positive
Curtain gas (CUR) : Nitrogen set at 15.00 (arbitrary units)
Temperature (TEM) : 550 °C
Ionspray voltage : 5500 V
Collision gas setting (CAD) : Medium
Gas 1 (GS1) : Air set at 40 (arbitrary units)
Gas 2 (GS2) : Air set at 40 (arbitrary units)
Interface heater (ihe) : On
Scan type : MRM

<table>
<thead>
<tr>
<th>MRM Conditions</th>
<th>Acibenzolar-S-Methyl primary transition</th>
<th>Acibenzolar-S-Methyl confirmatory transition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1 m/z</td>
<td>210.97</td>
<td>210.97</td>
</tr>
<tr>
<td>Q3 m/z</td>
<td>135.9</td>
<td>91.00</td>
</tr>
<tr>
<td>Dwell time</td>
<td>250 ms</td>
<td>250 ms</td>
</tr>
<tr>
<td>Resolution Q1</td>
<td>Unit</td>
<td>Unit</td>
</tr>
<tr>
<td>Resolution Q3</td>
<td>Unit</td>
<td>Unit</td>
</tr>
<tr>
<td>Declustering potential (DP)</td>
<td>51 V</td>
<td>51 V</td>
</tr>
<tr>
<td>Entrance potential (EP)</td>
<td>10 V</td>
<td>10 V</td>
</tr>
<tr>
<td>Collision energy (CE)</td>
<td>39 V</td>
<td>27 V</td>
</tr>
<tr>
<td>Collision cell exit potential (CXP)</td>
<td>6 V</td>
<td>14 V</td>
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</table>
### 4.4 Mass Spectrometer Conditions for CGA210007

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interface</td>
<td>TurboIonSpray</td>
</tr>
<tr>
<td>Polarity</td>
<td>Negative</td>
</tr>
<tr>
<td>Curtain gas (CUR)</td>
<td>Nitrogen set at 15.00 (arbitrary units)</td>
</tr>
<tr>
<td>Temperature (TEM)</td>
<td>550 °C</td>
</tr>
<tr>
<td>Ionspray</td>
<td>5500 V</td>
</tr>
<tr>
<td>Collision gas setting (CAD)</td>
<td>Medium</td>
</tr>
<tr>
<td>Gas 1 (GS1)</td>
<td>Air set at 40 (arbitrary units)</td>
</tr>
<tr>
<td>Gas 2 (GS2)</td>
<td>Air set at 40 (arbitrary units)</td>
</tr>
<tr>
<td>Interface heater (ihe)</td>
<td>On</td>
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<tr>
<td>Scan type</td>
<td>MRM</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MRM Conditions</th>
<th>CGA210007 primary transition</th>
<th>CGA210007 confirmatory transition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1 m/z</td>
<td>178.83</td>
<td>178.83</td>
</tr>
<tr>
<td>Q3 m/z</td>
<td>107.00</td>
<td>57.00</td>
</tr>
<tr>
<td>Dwell time</td>
<td>250 ms</td>
<td>250 ms</td>
</tr>
<tr>
<td>Resolution Q1</td>
<td>Unit</td>
<td>Unit</td>
</tr>
<tr>
<td>Resolution Q3</td>
<td>Unit</td>
<td>Unit</td>
</tr>
<tr>
<td>Declustering potential (DP)</td>
<td>-80 V</td>
<td>-80 V</td>
</tr>
<tr>
<td>Entrance potential (EP)</td>
<td>-10 V</td>
<td>-10 V</td>
</tr>
<tr>
<td>Collision energy (CE)</td>
<td>-26 V</td>
<td>-52 V</td>
</tr>
<tr>
<td>Collision cell exit potential (CXP)</td>
<td>-11 V</td>
<td>-5 V</td>
</tr>
</tbody>
</table>

Typical LC-MS/MS chromatograms from analysis of water samples are shown in the Figures Section.

### 4.5 Confirmatory Procedures for Acibenzolar-S-methyl and CGA210007

Final determination by LC-MS/MS with two transitions is considered to be highly specific; hence no further confirmatory conditions are included.
5.0 CALCULATION OF RESULTS

5.1 Multi-Point Calibration Procedure

Acibenzolar-S-methyl and CGA210007 residues may be calculated in μg/L for each sample as follows.

a) Prepare standard solutions over a concentration range appropriate to the expected residues in the samples (e.g. 30% LOQ to at least 20% above the highest fortified level as a minimum). An appropriate number of different concentrations within this range should be prepared (at least five).

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to acibenzolar-S-methyl and CGA210007. Calibration standard solutions should be interspersed throughout the analysis, after a maximum of four injections of sample solutions.

c) Generate calibration curve parameters using an appropriate regression package.

d) The following equation can be rearranged and used to calculate residues as follows:

\[ y = mx + c \]

Where \( y \) is the instrument response value, \( x \) is the standard concentration, \( m \) is the gradient of the line of best fit (“X-variable 1” in MS Excel) and \( c \) is the intercept value. An example of this equation generated using the experimental values of \( m \) and \( c \) should be included in the raw data, as should the “R-Squared” value for the regression.

Re-arrangement for \( x \) gives

\[ x = \frac{y - c}{m} \]

e) Calculate the acibenzolar-S-methyl and CGA210007 residues in the sample, expressed as μg/L, as follows

\[ \text{Residue (μg/L)} = \frac{\text{Analyte found (μg/mL)}}{\text{Sample conc. (L/mL)}} \]

Where analyte found (μg/mL) is calculated from the standard calibration curve and sample conc. is the final sample concentration in L/mL.
If residues need to be corrected for average percentage recovery e.g. for storage stability studies, then the equation below should be used.

\[
\text{Corrected Residue} = \frac{\text{Residue} \times 100}{\text{Average percentage Recovery}} \ (\mu g/L)
\]

5.2 Single-Point Calibration Procedure

Acibenzolar-S-methyl and CGA210007 residues may be calculated in \( \mu g/L \) for each sample using a mean standard response from each of the injections bracketing the sample as follows.

a) Make repeated injections of a standard containing acibenzolar-S-methyl and CGA210007 at an appropriate concentration into the LC-MS/MS operated under conditions as described in Section 4. When a consistent response is obtained, measure the peak areas obtained for acibenzolar-S-methyl and CGA210007.

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to acibenzolar-S-methyl and CGA210007.

c) Re-inject the standard solution after a maximum of four injections of sample solutions.

d) Calculate the acibenzolar-S-methyl and CGA210007 residues in the sample, expressed as \( \mu g/L \) using a mean standard response from each of the injections bracketing the sample as follows.

\[
\text{Residue (} \mu g/L) = \frac{\text{PK area (SA)}}{\text{PK area (STD)}} \times \frac{\text{Standard Conc.}}{\text{Sample Conc.}}
\]

Peak response for sample
Average peak response for bracketing standards
Concentration of standard (\( \mu g/mL \))
Sample concentration (L/mL)

If residues need to be corrected for average percentage recovery e.g. for storage stability studies, then the equation below should be used.

\[
\text{Corrected Residue} = \frac{\text{Residue} \times 100}{\text{Average percentage Recovery}} \ (\mu g/L)
\]

Although single point calibration may be used to quantify residues it is recommended that a calibration curve is generated with each analytical run to demonstrate the linearity of instrument response (Reference 4).
6.0 CONTROL AND RECOVERY SAMPLES

Control samples should be analyzed with each set of samples to verify the sample used to prepare recovery samples is free from contamination. A minimum of one control should be analyzed with each batch of samples.

At least two recovery samples (control samples accurately fortified with known amounts of analyte), including one at the method LOQ and one at the expected residue level, should also be analyzed alongside each set of samples. Provided the recovery values are acceptable they may be used to correct any residues found in the sample.

Recovery efficiency is generally considered acceptable when the mean values are between 70% and 110% and with a relative standard deviation of \( \leq 20\% \). 

When the method is used for monitoring purposes, control and recovery samples are not required where suitable control samples are not available.

7.0 SPECIFICITY

It is recommended that reagent blank samples be included in a sample set if contamination is suspected.

7.1 Matrix

LC-MS/MS is a highly specific detection technique. Interference arising from the matrices tested has not been observed.

7.2 Reagent and Solvent Interference

Using high purity solvents and reagents no interference has been found.

7.3 Labware Interference

This method uses mainly disposable labware. All reusable glassware should be detergent washed and then rinsed with HPLC grade methanol, acetone or acetonitrile prior to use.
CHEMICAL STRUCTURES

FIGURE 1  Acibenzolar-S-methyl

Compound Code Number : CGA245704
CAS Number : 135158-54-2
IUPAC Name : benzo[1,2,3]thiadiazole-7-carbothioic acid S-methyl ester
Molecular Formula : C_8H_6N_2OS_2
Molecular Weight : 210.3 g/mol
<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound Code Number</td>
<td>CGA210007</td>
</tr>
<tr>
<td>CAS Number</td>
<td>35272-27-6</td>
</tr>
<tr>
<td>IUPAC Name</td>
<td>benzo[1,2,3]thiadiazole-7-carboxylic acid</td>
</tr>
<tr>
<td>Molecular Formula</td>
<td>C$_7$H$_4$N$_2$O$_2$S</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>180.2 g/mol</td>
</tr>
</tbody>
</table>

![Molecular structure of CGA210007](image)
## APPENDIX 1  Apparatus

### Recommended Suppliers

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Description</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>General glassware</td>
<td>General glassware</td>
<td>Thermofisher</td>
</tr>
<tr>
<td>LC-MS/MS system</td>
<td>AB Sciex QTrap 5500 equipped with a TurboIonSpray source</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>HPLC system</td>
<td>Agilent 1290 binary pump, autosampler and column oven</td>
<td>Agilent</td>
</tr>
<tr>
<td>HPLC column</td>
<td>Water X-Select 50 x 3 mm 2.5 µm. Part number 186006105</td>
<td>Waters</td>
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</table>
APPENDIX 2 Reagents

Recommended Suppliers

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultra-pure water</td>
<td>HPLC grade</td>
<td>Thermofisher</td>
</tr>
<tr>
<td>Methanol</td>
<td>HPLC grade</td>
<td>Thermofisher</td>
</tr>
<tr>
<td>Formic acid</td>
<td>Analytical grade</td>
<td>Thermofisher</td>
</tr>
<tr>
<td>Acibenzolar-S-methyl and</td>
<td>GLP certified</td>
<td>Syngenta Crop Protection, Inc., P.O. Box</td>
</tr>
<tr>
<td>CGA210007</td>
<td></td>
<td>18300, Greensboro, NC 27419-8300.</td>
</tr>
</tbody>
</table>

Preparation of Reagents

a) 0.1% formic acid in ultra-pure water
   Add 1 mL concentrated formic acid to ultra-pure water in a 1 L volumetric flask.
   Adjust to the 1L mark with ultra-pure water. Stopper the flask securely and shake to mix thoroughly.
APPENDIX 3    LC-MS/MS Tuning Procedure

Calibration of Instrument

The instrument must be mass calibrated on a regular basis using polypropylene glycol (PPG) solutions according to the manufacturer’s instructions. Calibrate both mass resolving quadrupoles (Q1 and Q3).

Tuning Instrument for Acibenzolar-S-methyl and CGA210007

Infuse standard solutions of acibenzolar-S-methyl and CGA210007 (0.1 to 1.0 µg/mL) in mobile phase (see section 4) directly into the mass spectrometer interface at a rate at of approximately 10-20 µL/min. Roughly adjust interface parameters (sprayer position, spray, heater/auxiliary gas flows, as well as voltages of spray, orifice, and focusing ring) for a sufficiently high parent ion signal at \( m/z \) 211 for acibenzolar-S-methyl in positive ionisation mode and \( m/z \) 179 for CGA210007.

Using the Analyst software quantitative optimisation routine, tune the instrument for acibenzolar-S-methyl and CGA210007, ensuring that the correct ion is selected. If desired, manual tuning of the ion optics and collision energy can be carried out to ensure maximum sensitivity.

Finally, connect the LC-pump via the autosampler directly to the MS/MS instrument. Perform repetitive flow injection of an acibenzolar-S-methyl and CGA210007 standards using mobile phase at the flow rate to be used. Tune the interface parameters (sprayer position, spray and heater gas flows, spray, orifice, and focusing ring voltages) and the collision gas flow for maximum sensitivity.

For acibenzolar-S-methyl, in positive ionisation mode, the protonated molecular ion generated in the ion source \( (m/z \) 211) is selected and subjected to further fragmentation by collisional activation. The two most sensitive daughter ions \( (m/z \) 136 and \( m/z \) 91) are then selected and used for quantitative analysis. The \( m/z \) 136 fragment corresponds to loss of the COSMe side chain. The \( m/z \) 91 fragment corresponds to further loss of N-S leaving a phenyl amino fragment.

For CGA210007, in negative ionisation mode, the deprotonated molecular ion generated in the ion source \( (m/z \) 179) is selected and subjected to further fragmentation by collisional activation. The two most sensitive daughter ions \( (m/z \) 107 and \( m/z \) 57) are then selected and used for quantitative analysis. The \( m/z \) 107 fragment corresponds to loss of CO₂ and N₂ from the deprotonated molecular ion. The \( m/z \) 57 fragment corresponds to a rearrangement to give

\[
\begin{array}{c}
\text{N} \\
\text{O} \\
\text{N} \\
\text{N}
\end{array}
\]
Measure 20 mL of water into a 50 mL polypropylene centrifuge tube

Add 20 µL of concentrated formic acid to the sample and mix well by shaking

Fortify

Vortex or shake for vigorously for 10 seconds to mix well

Centrifuge or filter samples using a 0.45 µm syringe filter (if particles are visible)

Transfer 1.5 mL of an aliquot into an autosampler amber vial for LC-MS/MS analysis using matrix-matched calibration standards