# Simultaneous Determination of 21 Plant Growth Regulators in Various Fruits Using QuEChERS Coupled with an HPLC-MS/MS Technique

Application Note

Food


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## Abstract

This application note describes a method for the simultaneous detection of 21 plant growth regulators in fruits by QuEChERS combined with an HPLC-ESI-MS/MS technique based on our previous work [1]. The samples were initially extracted with acetonitrile containing 1 % acetic acid, followed by cleanup using C18 sorbent in the presence of magnesium sulfate. The resultant solution was separated on a C18 column using an Agilent Infinity UHPLC 1290 System, and detected with Agilent ESI-Triple Quadrupole Mass Spectrometry under multiple reaction monitoring (MRM) mode. The matrix-matched external standard calibration approach was used for quantitative analysis. All 21 compounds showed a linear dynamic range of 2–3 orders of magnitude in the 0.10–1,000 µg/L range for the examined matrixes of apple, pear, strawberry, grape, and orange, with correlation coefficents above 0.99. The limits of detection (LOD) and the limits of quantification (LOQ) of the method ranged between 0.020 µg/kg–6.0 µg/kg and 0.10 µg/kg–15.0 µg/kg, respectively. For all the compounds, the average spiked recoveries ranged from 73.0 % to 111.0 %, and the relative standard deviations (RSDs, n = 6) were in the range of 3.0–17.2 %. The method is quick, easy, effective, sensitive, and accurate. It meets the requirements of the determination of these plant growth regulators in fruits.



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## Introduction

Plant growth regulators (PGRs) are generally synthesized compounds with similar structures and physiological functions to native and endogeneous plant hormones. They can play key roles in enhancing plant stress resistance, promoting plant cellular division and growth, as well as improving plant yield and quality. As a result, they have been widely used in agricultural production. However, overuse of these PGRs without supervision may lead to elevated levels of PGR residues in vegetables and fruits. This can present a threat to public health. Currently, a number of countries and international organizations have enacted regulations by setting up the maximum allowable residue levels (MRLs) of some PGRs in various fruits. The European Union has regulated daminozide, thidiazuron, and triapenthenol in most fresh fruits with MRLs of 0.02, 0.01, and 0.01 mg/kg, respectively [2]. Japan limits the MRL of trinexapac-ethyl at 0.02 mg/kg in watermelon [3]; China limits the MRLs of forchlorfenuron in grape at 0.05 mg/kg and 2,4-D in some fruit such as apple and pear at 0.01 mg/kg [4]. Hence, it is essential to develop a sensitive method to monitor these PGR residues in related agricultural products.

LC/MS has been proposed to replace traditional methods such as ELISA, GC, and LC methods for the determination of PGR residues [5-7]. There are multiple classes of PGRs that vary dramatically in chemical structures. This makes simultaneous analysis of these PGRs quite challenging. Most reported LC/MS methods only focus on several classes of PGRs. Here, we are trying to develop a universal method that allows simultaneous determination of the commonly used PGRs in various fruits by combining the QuEChERS procedure with the HPLC-MS/MS technique.

## Experimental

### Reagents and materials

The 21 plant growth regulators (PGRs) include daminozide, chlormequat chloride, mepiquat chloride, choline chloride, 2,4,5-triiodobenzoic acid, gibberellic acid, cyclanilide, abscisic acid (ABA), forchlorfenuron, 6-benzylaminopurine, thidiazuron, 2,4-D, cloprop, 4-chlorophenoxyacetic acid (4-CPA), 1-naphthylacetic acid, indole-3-acetic acid (IAA), inabenfide, paclobutrazol, uniconazole, triapenthenol, and trinexapac-ethyl, all were purchased from Dr. Ehrensterfer (Germany) with purity higher than 96 %.

The standard compounds were prepared in methanol (HPLC grade) at a concentration of 100.0 mg/L, and stored at 4 °C. The stock solutions were further diluted using initial mobile phase or matrix extract in a series of concentrations for external calibration.

### Sample preparation

Ten grams of sample was accurately weighted into a 50-mL centrifuge tube. Then, 10 mL of acidified acetonitrile (1 % acetic acid in acetonitrile) was added. The mixture was homogenized for 2 minutes. Four grams of $MgSO_4$ and 1.5 g of sodium acetate were added to the vial. It was then votexed for 1 minute, followed by centrifugation at 4,000 rpm for 3 minutes. Two milliliters of the supernatant sample solution was loaded into a clean 2-mL vial containing 25-mg C18 sorbent and 150-mg anhydrous $MgSO_4$. The vial was votexed for 1 minute, and then centrifuged for 5 minutes at 16,000 rpm. The supernatant was filtered through a 0.22-µm memebrane, and stored at 4 °C before LC-MS/MS analysis.

### Instrumentation conditions

**LC configuration and conditions**

- Agilent Infinity UHPLC 1290 binary pump (G4220A)
- Agilent High performance AutoSampler (G4226A)
- Agilent AutoSampler ThermoStat (G1330B)
- Agilent Thermostatted Column Compartment SL (G1316B)

| | |
|---|---|
| Column | Agilent XDB-C18, 4.6 × 100 mm, 5.0 µm |
| Column temperature | 35 °C |
| Injection volume | 5 µL |
| Needle wash | Flushport (100 % methanol), 5 seconds |
| Mobile phase | A) 5 mM Ammonium acetate/0.05 % formic acid in water<br>B) Acetonitrile |
| Gradient flow rate | 0.5 mL/min |

Gradient elution profile is shown in Table 1

*Table 1. The Gradient Elution Profile*

| Time | Sol. A (%) | Sol. B (%) |
|---|---|---|
| 0 | 95 | 5 |
| 2 | 95 | 5 |
| 12 | 20 | 80 |
| 18 | 20 | 80 |
| 18.1 | 95 | 5 |
| 24 | 95 | 5 |

**MS configuration and conditions**

- Agilent 6460 Triple Quadrupole Mass Spectrometer with Agilent Jet Stream ionization source

| | |
|---|---|
| Ionization mode | positive/negative ionizations |
| Scanning mode | Multiple reaction monitoring (MRM) |
| Capillary voltage | 4,000 V |
| Nozzle voltage | 500 V |
| Nebulizer pressure | 45 psi |
| Dry gas temperature | 300 °C |
| Dry gas flow rate | 10 L/min |
| Sheath gas temperature | 375 °C |
| Sheath gas flow rate | 11 L/min |

# Results and Discussion

## Optimization of MS detection

Initially, the MS acquisition conditions were optimized by infusion of a standard solution of each compound into the mass spectrometer. Stable precursor ions such as $[M+H]^+$ under positive ionization, or $[M-H]^-$ under negative ionization were selected for further fragmentation. Some compounds such as 6-BA, thidiazuron, forchlorfenuron, and inabenfide can produce both $[M+H]^+$ and $[M-H]^-$. Considering the nature of the compounds and the sensitivity as well as necessity of time segment scanning, 6-BA, thidiazuron, and forchlorfenuron were detected under negative mode, whereas inabenfide was detected under positive mode. With the selection of precursor ions, the fragmenor voltage was then optimized to the highest sensitivity for precusor ions. Product ion scanning and MRM scanning were conducted to obtain specific fragment ions and the collision energies in favor of highest response and least interference. The optimized MRM transitions and the corresponding parameters are shown in Table 2.

*Table 2. MS/MS Parameters of 21 PGRs*
*Note: * Represents the Quantitative Ions*

| Analyte | Parent ion (*m/z*) | Product ion (*m/z*) | CE (V) | Fragment (V) |
|---|---|---|---|---|
| **0–5 minutes** | | | | |
| Choline choride | (+) 104. 1 | 45.1<br>60.2* | 23<br>17 | 100 |
| Chlormequat chloride | (+) 122.1 | 58.2*<br>63.1 | 33<br>21 | 130 |
| Mepiquat choride | (+) 114.2 | 58.2<br>98.1* | 29<br>28 | 140 |
| Daminozide | (+) 161.1 | 143.1*<br>61.2 | 8<br>10 | 90 |
| **5–13 minutes** | | | | |
| Gibberellic acid | (–) 344.9 | 143.1*<br>239.2 | 25<br>8 | 170 |
| 6-Benzylaminopurine | (–) 224.1 | 133.0*<br>106.2 | 21<br>36 | 140 |
| IAA | (–) 174.0 | 130.1*<br>128.1 | 7<br>20 | 70 |
| ABA | (–) 263.2 | 153.1*<br>204 | 2<br>12 | 120 |
| 1-Naphthylacetic acid | (–) 185.0 | 141.3* | 4 | 100 |
| Thidiazuron | (–) 219.1 | 100.1*<br>71.2 | 4<br>35 | 80 |
| 2,4-D | (–) 219.0 | 161.0*<br>125.0 | 8<br>25 | 100 |
| Cloprop | (–) 199.1 | 70.9<br>127.0* | 4<br>10 | 80 |
| 2,3,5-Triiodobenzoic acid | (–) 498.8 | 126.8<br>454.8* | 18<br>1 | 45 |
| 4-chlorophenoxyacetic acid | (–) 185.0<br>(–) 187.0 | 127.1*<br>129 | 10<br>10 | 100 |
| Forchlorfenuron | (–) 246.1 | 91.1<br>127.1* | 26<br>6 | 130 |
| Cyclanilide | (–) 272.0 | 160.0*<br>227.9 | 15<br>2 | 100 |
| **13–24 minutes** | | | | |
| Inabenfide | (+) 339.2 | 321.2*<br>80.2 | 17<br>33 | 140 |
| Trinexapac-ethyl | (+) 253.5 | 69.2*<br>185.3 | 17<br>7 | 70 |
| Paclobutrazol | (+) 294.1 | 70.1*<br>125 | 21<br>44 | 120 |
| Uniconazole | (+) 292.1 | 70.1*<br>124.9 | 26<br>35 | 110 |
| Triapenthenol | (+) 264.2 | 67.1<br>70.1* | 37<br>25 | 70 |

## Selection of LC conditions

Narrow bore and small particle columns were initially used for the separation of 21 PGRs. However, due to the wide polarity differences of the 21 PGRs, very polar compounds such as daminozide, chlormequat chloride, mepiquat chloride, and choline chloride eluted out of column within 2 minutes, and the coeluting interference suppression during ESI-MS/MS detection was very severe. An alternative column, Agilent XDB-C18 (4.6 × 150 mm, 5 µm), was then used. As a result, retention times higher than 2.5 minutes for all compounds were achieved, and the coeluting interference from the matrixes was moderately reduced. Hence, an Agilent XDB-C18 column was selected for the separation of the target compounds.

A binary mobile phase of acetonitrile/water was initially selected. To reach better separation and detection sensitivity, formic acid, ammonium acetate, or the mixture of formic acid-ammonium acetate were often added to the aqueous phase to modulate the separation selectivity and sensitivity. By comparing three types of mobile phase compositions, it was found that the mixture of formic acid and ammonium acetate at a concentration of 5 mmol/L and 0.05 % respectively showed the best peak resolution and peak shape, and was selected as the aqueous phase.

Under the optimized MS and LC conditions, the typical overlapped MRM chromatograms for the 21 PGRs are shown in Figure 1.

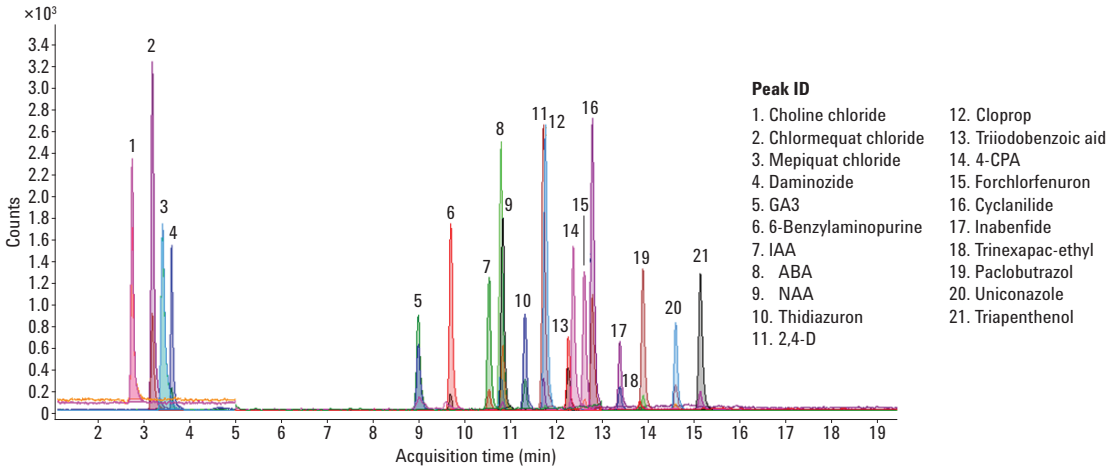


*Figure 1. The typical overlapped MRM chromatograms for 21 PGRs.*

## Optimization of QuEChERS extraction and cleanup Procedure

The QuEChERS protocol was applied for sample extraction and cleanup. To better extract the target analytes from fruit matrixes, acetonitrile, acetonitrile/1 % acetic acid, acetonitrile/2 % acetic acid, and acetonitrile/1 % NaOH were compared. As shown in Figure 2, the recoveries for all 21 PGRs spiked in the apple matrix can reach 80 % or higher when using acetonitrile/1 % acetic acid. As a result, acetonitrile/1 % acetic acid was selected as the extraction solvent.

For further cleanup, dispersive solid phase extraction (dSPE) using classic sorbents such as PSA, C18, GCB, or their combinations in the presence of $MgSO_4$ were examined. As shown in Figure 3, with PSA as the cleanup sorbent, only five compounds including forchlorfenuron, inabenfide, paclobutrazol, uniconazole, and triapenthenol showed recoveries higher than 70 %, but all other compounds showed recoveries below 40 %. Combining PSA with C18 or GCB did not show any improvement. In comparison, using C18 in the presence of $MgSO_4$ as the cleanup sorbent, 21 analytes were recovered efficiently with recovery values all higher than 75 %. Therefore, C18 was selected for cleanup.

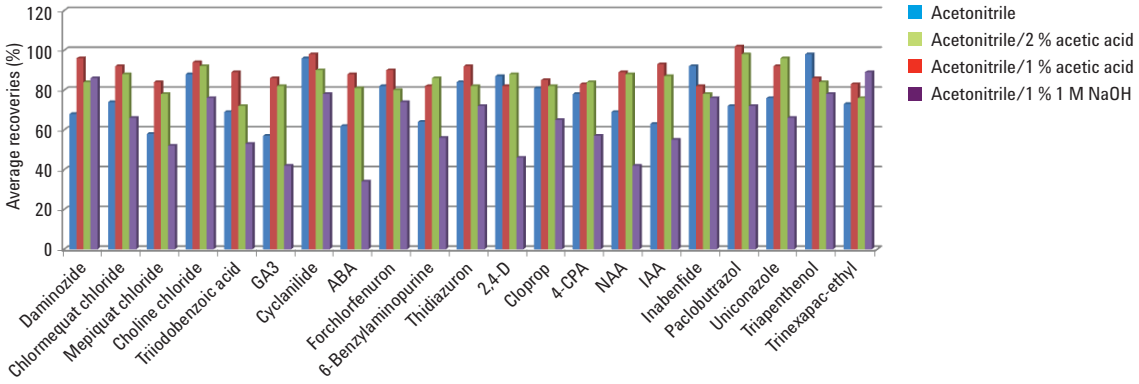


*Figure 2. Effect of different solvents on the extraction efficiency of the analytes.*

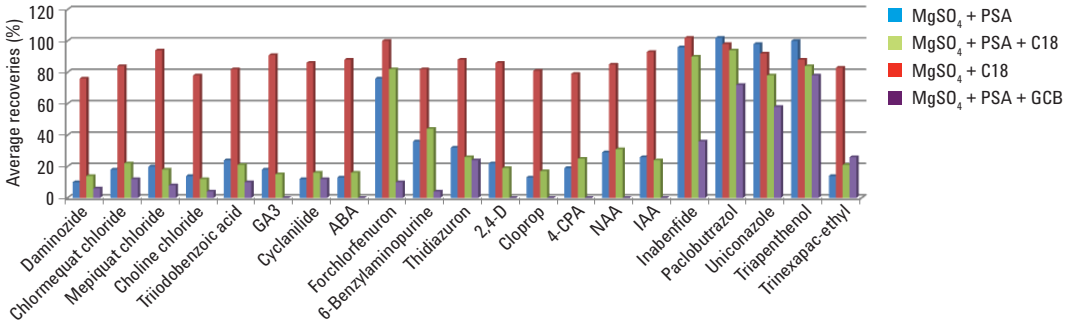


*Figure 3. Effects of different sorbents on the recovery of analytes.*

## Method performance

Matrix interference is not negligible during LC/MS analysis, which can either enhance or suppress the response of target analytes, leading to decreased quantitation accuracy. To improve analysis accuracy, matrix interference has to be evaluated, and proper strategy has to be applied to minimize the matrix effect. Here we used the relative response to evaluate the matrix effect (matrix effect ME% = peak area in matrix/peak area in pure solvent × 100 %, where matrix enhancement is present when ME% > 100 %, and matrix suppression is present when ME% < 100 %). As shown in Figure 4, 48 % of compounds showed that ME% exceeded the acceptable range of 80–120 % assigned by most regulation bodies. Particularly, apple and orange matrixes showed matrix enhancement and suppression effects for approximately one third of compounds, respectively. It indicated that the matrix effect cannot be ignored during analysis.

To minimize the quantitation bias due to matrix effects, a matrix-matched external standard calibration method was used in this study. As shown in Figure 5, excellent linearity can be achieved in the examined range for each compound with correlation coefficients higher than 0.99. LOD (3 × S/N) and LOQ (10 × S/N) of the method were determined from the chromatogram at the lowest matrix-matched solution for each compound, which are shown in Table 3. As demonstrated, all compounds have LOQs significantly lower than the current available MRLs specified by the regulation bodies, indicating that the method is highly sensitive and meets regulation requirements.

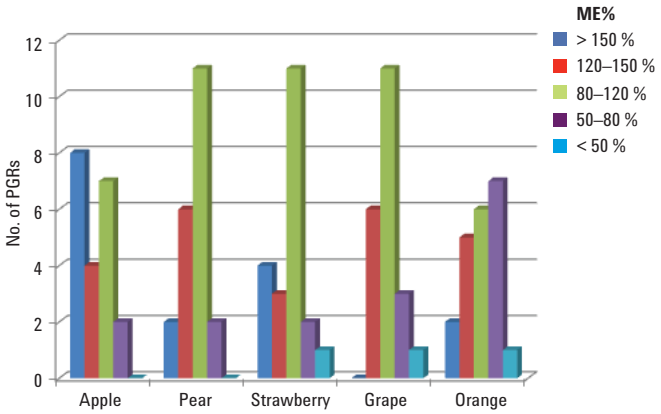


*Figure 4. Distribution of matrix effects (represented as ME%) for 21 PGRs in five fruit matrixes.*

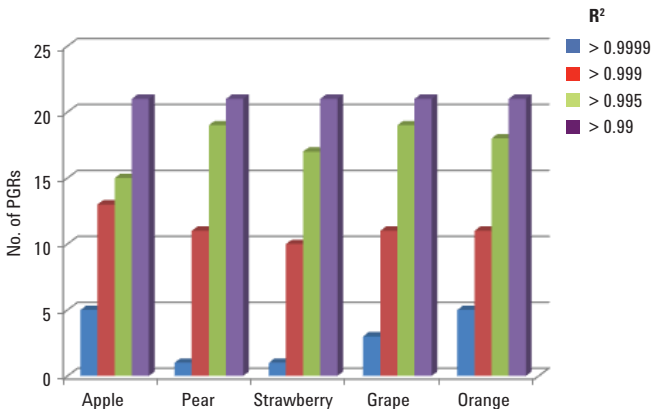


*Figure 5. The linearity ($R^2$) of matrix-matched calibration curves in five fruit matrixes.*

*Table 3. LOD and LOQ of the Method in Five Fruit Matrixes*

| Name | LOD (µg/kg) | LOQ (µg/kg) | Name | LOD (µg/kg) | LOQ (µg/kg) |
|---|---|---|---|---|---|
| Choline chloride | 0.050 | 0.30 | Cloprop | 1.5 | 8.0 |
| Chlormequat chloride | 0.050 | 0.20 | Triiodobenzoic acid | 1.0 | 5.0 |
| Mepiquat chloride | 0.050 | 0.30 | 4-CPA | 1.0 | 5.0 |
| Daminozide | 0.50 | 2.0 | Forchlorfenuron | 0.020 | 0.10 |
| GA3 | 6.0 | 15.0 | Cyclanilide | 0.040 | 0.20 |
| 6-Benzylaminopurine | 0.80 | 3.0 | Inabenfide | 0.050 | 0.20 |
| IAA | 5.0 | 15.0 | Trinexapac-ethyl | 1.4 | 5.0 |
| ABA | 1.5 | 5.0 | Paclobutrazol | 0.020 | 0.10 |
| NAA | 4.0 | 10.0 | Uniconazole | 0.080 | 0.80 |
| Thidiazuron | 0.050 | 0.80 | Triapenthenol | 0.040 | 0.40 |
| 2,4-D | 1.2 | 5.0 | | | |

## Accuracy and precision

To further evaluate the accuracy and precision of this method, a variety of samples including apple, pear, strawberry, grape, and orange were tested. Those that showed no detectable target analytes were selected as the blank matrixes. Three levels of each analyte were spiked into the blank matrixes with six replicates, and the spiked samples were then subjected to extraction and cleanup, followed by LC/MS/MS analysis. As shown in Figure 6, the recoveries for the 21 analytes in the five blank matrixes were all within 73.0–111.0 %, with relative standard deviation (RSD) within 3.0–17.2 %. Such accuracy and precision meets the requirement for residue analysis.

## Conclusions

This application note demonstrates a method for the simultaneous quantitation of 21 plant growth regulators. With matrix-matched calibration, the developed method has wide dynamic range, with correlation coefficents higher than 0.99. It is accurate and robust, with the spiking recoveries within 73.0–111.0 %, and the RSD within 3.0–17.2 %. Moreover, it is sensitive, with the LOQ of the 21 PGRs ranging from 0.10 µg/kg to 15.0 µg/kg. This is significantly lower than the currently available determination methods and the regulation levels, hence it can be applied to the routine monitoring of these PGR residues in fruits.

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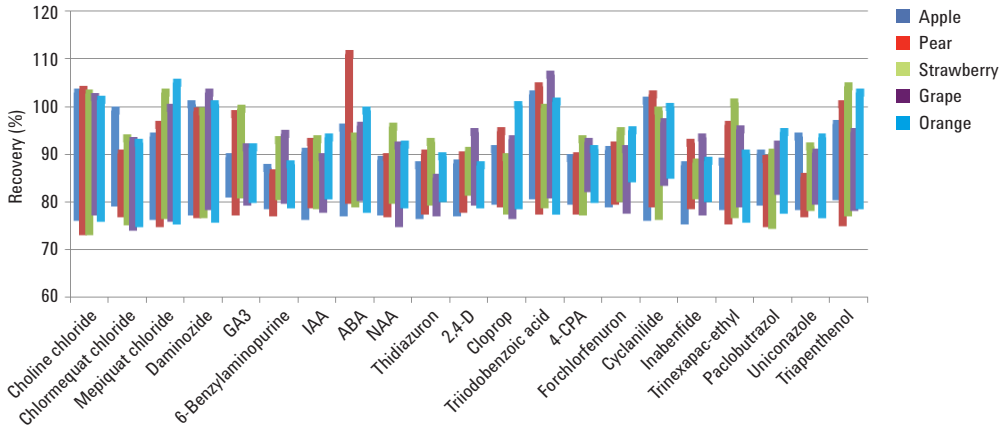


*Figure 6. Recoveries for the 21 analytes in the five blank matrixes.*

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