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BRIEF COMMUNICATION

A Rapid, Simple Method for the Determination of 6-Benzyladenine by Pyrolysis Mass Spectrometry

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Abstract Pyrolysis mass spectrometry (PyMS) is a rapid, simple, high-resolution analytical method based on thermal degradation of complex material in a vacuum. PyMS was used for the quantitative determination of 6-benzyladenine (BA) supplemented to agar-solidified culture media (ASM) in this study. When subjected to PyMS, pure BA generated prominent fingerprint peaks. The peaks at *m/z* 68 and 123 were chosen for the quantitative measurement of BA because of the highest signal among those generated from pure BA and because of one of the highest masses among those with a prominent signal, respectively. To establish a standard curve for BA concentration in ASM, the combined peak intensity at *m/z* 68 and 123 was plotted against BA concentration ranged from as low as 0.44 μM after logarithmic transformation of both parameters. A linear regression line was yielded, which indicates BA concentration in ASM is directly proportional to the peak intensity, with R^2=0.9052, significant at the 99% level. These results suggest that PyMS enables the quantitative determination of growth regulators and other related compounds in plant materials in a rapid, simple, sensitive, accurate manner.

Keywords 6-Benzyladenine · Linear regression · Pyrolysis mass spectrometry · Quantitative determination · Rapid method

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Introduction

Pyrolysis mass spectrometry (PyMS) is a rapid, simple, high-resolution method based on thermal degradation of complex material in a vacuum. PyMS has been widely applied to the discrimination of closely related microbial strains (Timmins et al. 1998). Recently, PyMS is also used for the discrimination of plant calluses based on embryogenic capacity (Kim et al. 2007) and taxonomic classification of higher plants (Kim et al. 2004, 2007) and genetic discrimination of *Catharanthus roseus* cultivars (Kim et al. 2009) as well.

In addition, PyMS enables the quantitative determination of the levels of chemical compounds in biological materials. PyMS determined the levels of indole produced from tryptophan in transformed strains of *Escherichia coli* (Goodacre and Kell 1993), the undecapeptide cyclosporin A in human blood (Ghassempour et al. 1999), the antibiotic ampicillin in mixtures of *E. coli* and *Staphylococcus aureus* (Goodacre et al. 1994), and casamino acids in mixtures with glycogen (Goodacre and Kell 1993).

Quantitative determination of plant growth regulators is often critical to provide molecular and/or physiological interpretation to plant growth and development. Determination of plant growth regulators has been carried out by high-pressure liquid chromatography (Sánchez et al. 1996), gas chromatography-mass spectrometry (Takatsuto 1994),

and immunoassays or radioimmunoassay (Weiler 1984), and more recently by liquid chromatography-electrospray ionization tandem mass spectrometry (Chiwocha et al. 2003) and capillary electrophoresis with spectrophotometric UV detection (Assunção et al. 2009). However, these techniques require time-consuming sample separation process.

In this study, we attempted to quantitatively determine 6-benzyladenine (BA) contained in agar-solidified medium (ASM) by PyMS and assessed whether this approach could be used in a rapid, simple, sensitive manner. BA is a synthetic cytokinin affecting plant growth and development by stimulating cell division. The role of cytokinin in plant growth and development has recently been reviewed by (Choi and Hwang 2007).

Materials and Methods

Agar-solidified Medium

A series of BA concentrations (0, 0.44, 1.33, 4.44, and 13.3 μM) were supplemented to Murashige and Skoog (MS; 1962) medium comprising MS salts, 100 mg L^{-1} myoinositol, 0.4 mg L^{-1} thiamine·HCL, 3% sucrose, and solidified with 0.4% Gelrite.

PyMS Analysis

Approximately 1 mm^3 agar blocks cut from agar-solidified media supplemented with a series of BA concentrations

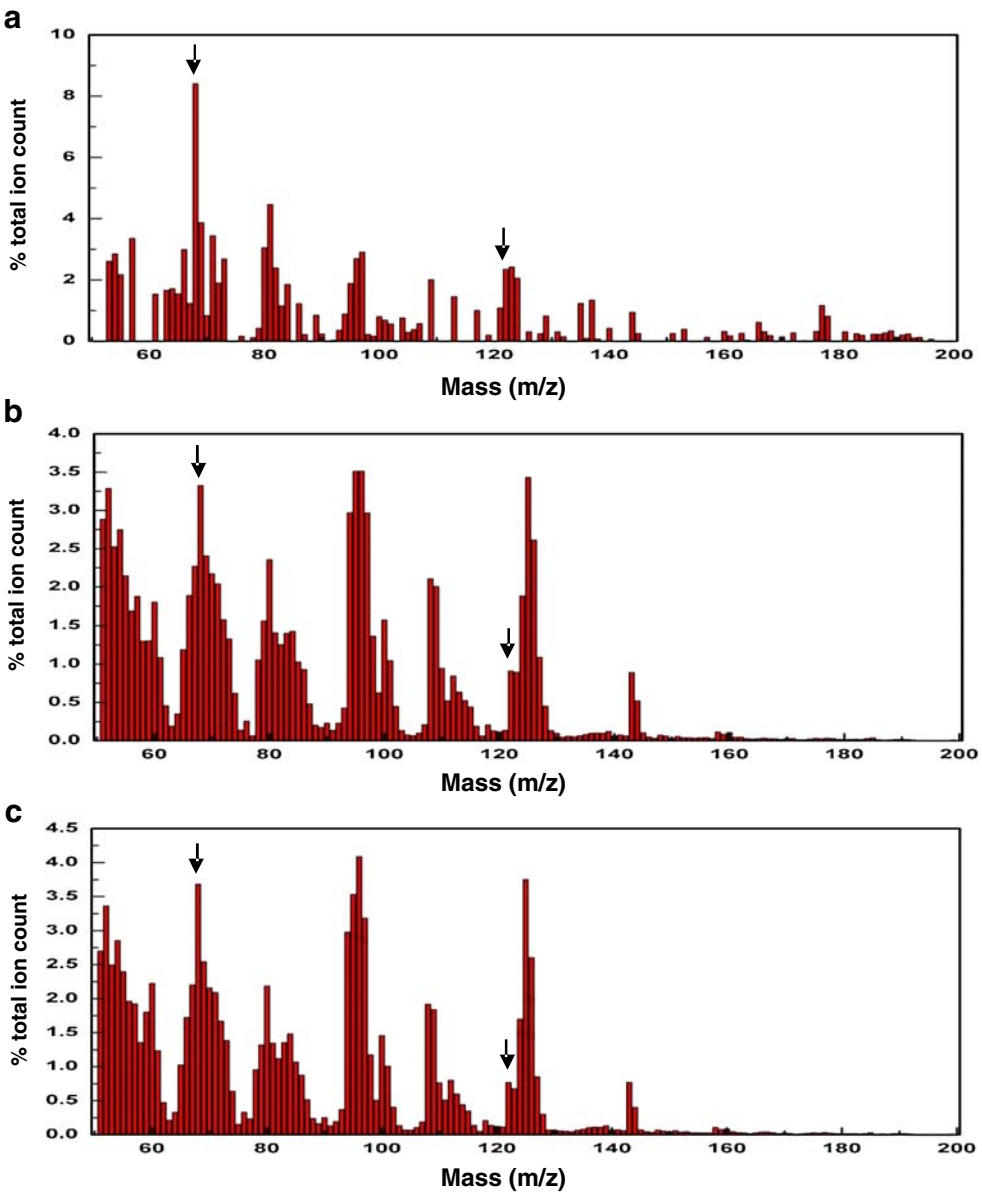

Fig. 1 Quantitative pyrolysis mass spectrum of BA (**a**), ASM (**b**), and ASM supplemented with 4.44 μM BA (**c**). *Arrows* indicate pyrolysates 68 (*left*) and 123 *m/z* (*right*)

were oven dried at 50°C for 30 min before PyMS analysis. Samples were run in triplicate for each treatment (different concentrations of BA). The pyrolysis mass spectrometer RAPyD-400 (mass range, 12–400 Da; ion counting to 25 MHz; SS Scientific Limited, England) was used. Curie-point pyrolysis was at 530°C for 3 s.

Statistical Analysis

Pyrolysate profiling data were normalized to percent total ion count to remove the influence of sample size before being subjected to Origin (version 8, OriginLab, USA) program for regression analysis. For logarithmic transformation, the zero concentration of BA was approximated with 0.04 μM, 2 log unit below the lowest "real" *x*-axis independent variable because the log of 0 is undefined.

Results and Discussion

The representative PyMS spectra of pure BA and ASM supplemented with BA are shown in Fig. 1. The *x*- and *y*-axes represent the mass-to-charge ratio (*m/z*) and the measurement of the ion count for any particular *m/z* value ranging from 51 to 200, respectively. Pure BA generated a spectrum that exhibited the most prominent peak at *m/z* 68 when subjected to PyMS (Fig. 1a). In addition, the peat at *m/z* 123 was one of the highest masses among those with a prominent signal. Therefore, the peaks at *m/z* 68 and 123 were chosen for the quantitative measurement of BA. Furthermore, for both ASM (Fig. 1b) and BA (Fig. 1a), the peak intensity of ASM+4.44 μM BA (Fig. 1c) at *m/z* 68 and 123 was additive. However, we do not rule out the existence of peaks that would provide more accurate measurements.

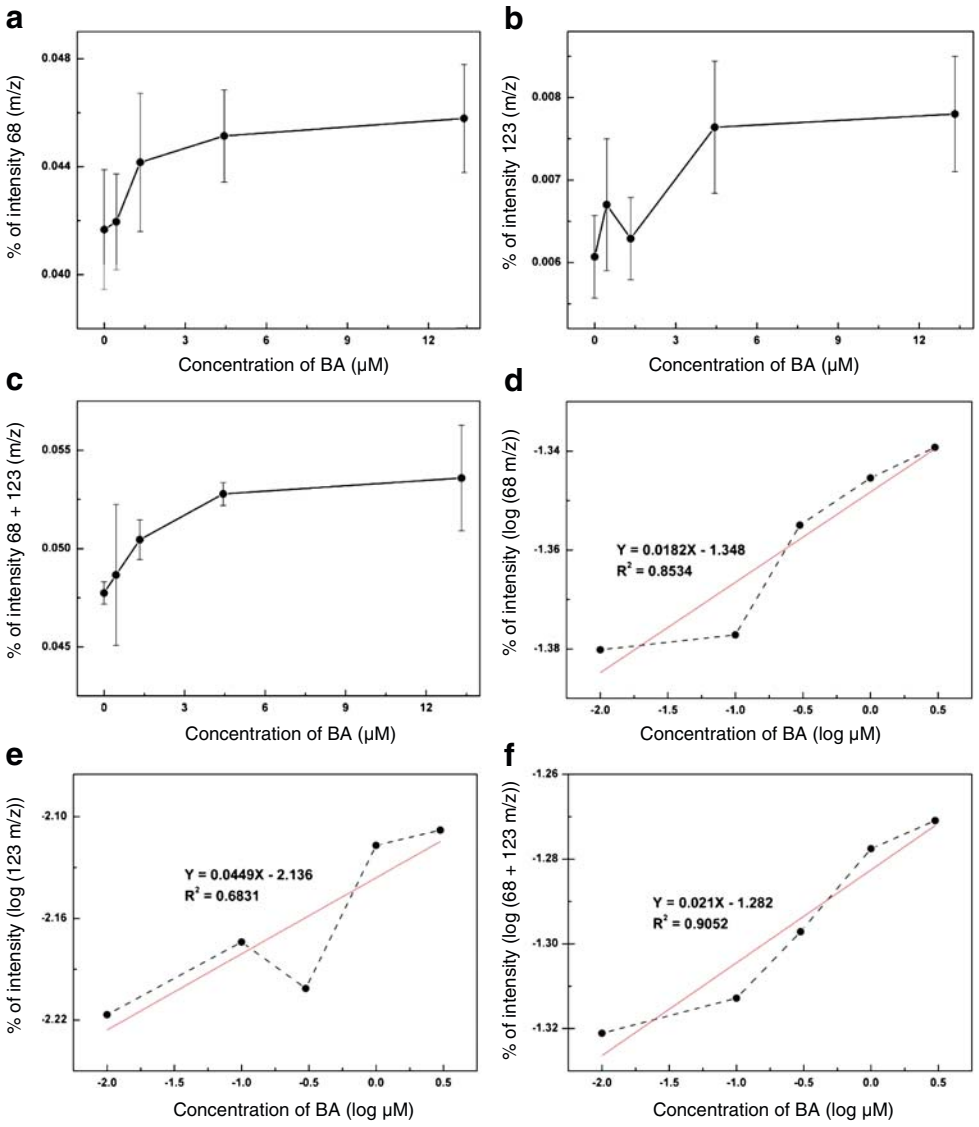

Fig. 2 Plots of the percent peak intensity against BA concentration at *m/z* 68, 123, and 68+123 and regression analysis of the plots after logarithmic transformation. **a**–**c** At *m/z* 68, 123, and 68+123, respectively. *Dots* indicate the mean values of the percent peak intensity at the given BA concentration. Three replicates were used. *Vertical bars* indicate standard deviation. **d**–**f** After logarithmic transformation of **a**, **b**, and **c**, respectively. R^2 indicates coefficient of determination

The peak intensity at *m/z* 68 showed a sigmoid curve when plotted against BA concentration: the total intensity was rather proportional to BA concentration at low levels and became plateau at higher levels (Fig. 2a). To generate a standard curve proportional to BA concentration, both of the observations of BA concentration (*x*-axis) peak intensity (*y*-axis) were logarithmically transformed and plotted. A logarithmic transformation enabled to convert a sigmoid curve into a linear regression line described by an equation with R^2 (coefficient of determination)=0.8534, which is statistically significant at the 99% level (Fig. 2d). In the same manner, the peak intensity at *m/z* 123 was plotted against BA concentration, showing a regression line with R^2=0.6831 (Fig. 2e), significant at the 99% level.

However, the combined peak intensity at *m/z* 68 and 123 resulted in an improved fit to a straight line, and the value of R^2 was enhanced to 0.9052 (Fig. 2f), significant at the 99% level. These results indicate that BA concentration in ASM can be determined with sensitivity and accuracy comparable to those of conventional analytical methods for the quantitative determination of plant growth regulators.

On the other hand, it would be necessary to determine a specific compound quantitatively based on more than two different fingerprint peaks in the spectrum of a sample, thereby enabling to confirm that peak intensities used for quantitative determination are not likely to have resulted from an overlap of common fingerprint peaks from two different compounds. In this study, the peak intensity at 68 *m/z* could be used for the determination of BA concentration in ASM and the peak intensity at 123 *m/z* for confirmation.

Artificial neural networks (ANNs) were used to determine concentrations of indole (Goodacre and Kell 1993), casamino acids (Goodacre et al. 1993), and cytochrome b_5 (Goodacre et al. 1994) contained in biological materials based on PyMS spectra. However, ANNs is an adaptive system to model complex relationships between inputs and outputs, tending to be a black box without analytical basis. In this study, we used a logarithmic transformation of the observations, expressing the relationship between BA concentration and the peak intensity with a linear regression equation.

Most of the current analytical methods for plant growth regulators or untargeted secondary metabolites (Kumar and Gupta 2008) are complicated and time-consuming, which are usually carried out by a combined process of the separation and detection of compounds. PyMS requires no separation process to save the time and a much smaller quantity of a sample for reliable detection as well. This approach could also be used for the quantitative determination of exogenous plant growth regulators in culture medium consumed by plant cultures.

PyMS was used for the quantitative determination of metabolites and proteins in various biological materials (Goodacre and Kell 1993; Goodacre et al. 1993; Ghassempour et al. 1999). However, PyMS has seldom been used in plant biology for quantification of compounds. In this study, low concentrations of BA were successfully determined by PyMS, suggesting that this method is extensively applicable to other plant growth regulators and secondary metabolites in plant materials.

In conclusion, we demonstrated that PyMS enabled to quantitatively determine BA concentration in ASM based on a linear regression between the fingerprint peak intensity and concentration of BA after logarithmic transformation of both parameters in this study, which was rapid, simple, sensitive, and accurate. Overall results suggest that PyMS may provide a means to quantitatively determine other plant growth regulators and secondary metabolites in plant materials in a high-throughput manner.

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