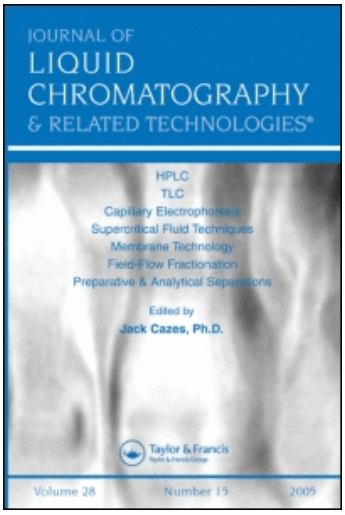

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HPLC Coupled with Atmospheric Pressure Chemical Ionization Mass Spectrometry for the Determination and Identification of 6-Benzylaminopurine, Fluocoumafen, and Brodifacoum in Fruits

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HPLC Coupled with Atmospheric Pressure Chemical Ionization Mass Spectrometry for the Determination and Identification of 6-Benzylaminopurine, Fluocoumafen, and Brodifacoum in Fruits

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Abstract: An improved method has been developed and validated to determine 6-benzylaminopurine (6-BAP) residue in fruits by high performance liquid chromatography coupled with an atmospheric pressure chemical ionization mass spectrometry (HPLC–APCI–MS) in the positive mode. The residue of 6-BAP was extracted by liquid–liquid extraction using acidified methanol to be isolated from the fruit matrix. The separation was carried out on a Zorbax SB C_{18} column (150 mm × 4.6 mm i.d., 5 μm) with 0.1% acetic acid/methanol (25/75, v/v) as mobile phase in isocratic mode. The calibration curves were linear ($r^2 > 0.998$) in the concentration range of 0.01 ∼ 1.00 mg/L with a lower limit of quantification of 2.0 ng/g for 6-BAP in apples, pears, and tomatoes. Intra-day and inter-day relative standard deviations (RSDs) were less than 6.3 and 8.5%, respectively. Recoveries of 6-BAP ranged from 84.7 ∼ 94.1%. This assay can be used to determine trace 6-BAP in apples, pears, and tomatoes.

Keywords: 6-Benzylaminopurine, Fruit, Apple, Pear, Tomato, HPLC–APCI–MS

INTRODUCTION

As a plant growth regulator, 6-benzylaminopurine (6-BAP) (Figure 1) is the first generation synthetic cytokinin. It can inhibit degradation of chlorophyll, nucleic acid, and protein; promote delivery of amino acid, inorganic salt, and growth regulators to applied positions. It helps plant to keep green and retard

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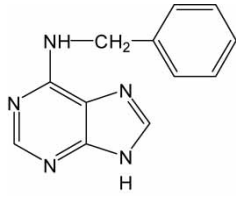

Figure 1. Chemical structure of 6-BAP.

aging. It can be used in agriculture, horticulture, for plants at different stages, from germination to harvest.[1–5] 6-BAP is more efficient and cost effective than isolating its natural counterpart, Cytokinin B, from plant sources. Today, 6-BAP is a common model compound for one of the most important classes of plant hormones—cytokinins, and has been widely used in the agricultural production in China. In order to further study the function of 6-BAP and better control the dosage and the residue in the course of the production, the development of a rapid, simple, and accurate method for the determination of 6-BAP residue in fruits is of great importance and interest for the agricultural industry.

So far, the most commonly used method for the determination of 6-BAP is high performance liquid chromatography (HPLC) with a UV detector.[6–7] However, the sensitivity of UV is limited, and not suitable for trace level residue of 6-BAP. Otherwise, electrochemical method was developed for the determination of 6-BAP in apples and tomatoes.[8] This method also suffered from a lack of sensitivity and selectivity, and is not sensitive enough for determining low level residue in the fruits. With the advent of ionization techniques, mass spectrometry (MS) has played an important role for the analyses of drugs because of its excellent specificity, speed, and sensitivity.[9–15] In this paper, we apply the atmospheric pressure chemical mass spectrometry (APCI-MS) to develop and validate a rapid, sensitive, and specific method in the selected ion monitoring (SIM) mode to determine trace 6-BAP residue in apples, pears, and tomatoes.

EXPERIMENTAL

HPLC-APCI-MS System

The analytical method for development and validation was performed on an Agilent 1100 series LC/MSD Trap SL System (Agilent Technologies, Germany), consisting of a quaternary pump (G1311A), a column thermostat (G1316A), a degasser unit (G1379A), an autosampler (G1313A), a diode array detector (G1315B), and an ion trap mass spectrometer with both atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI)

interfaces. The HPLC-MS system was controlled, and data were analyzed, on a computer equipped with LC/MSD Trap Software 4.2 (Bruker). All tubing used for the connection was PEEK (0.25 mm I.D., Agilent Technologies, Germany).

Solvents and Materials

Methanol (MeOH), acetic acid, and ethyl acetate (Merck, Darmstadt, Germany) used were HPLC grade. Water used was supplied by a Milli-Q water purification system from Millipore (Molsheim, France). 6-BAP (>99%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Uncontaminated apples, pears, and tomatoes were collected at the Best Market (Zhoushan, China); they were tested by an HPLC-APCI-MS method, and it was confirmed that no 6-BAP residue was contained in them. Ten samples, apples, pears and tomatoes, respectively, were collected from the farm product market in Zhoushan (Zhejiang, China). All samples were prepared by high speed mixing, in an IKA T25 blender (IKA, Guangzhou, China), and kept at -20°C until analysis.

Extraction of 6-BAP

Ultrasound assisted extraction was carried out in a KQ 500DB ultrasonic cleaning bath (Kunshan Ultrasonic, Kunshan, Jiangsu, China), which had a mean operating frequency of 40 KHz and a maximum peak power of 500 W. The extraction was performed at room temperature, and the power was adjusted to 200 W. The extraction protocol was as follows: 5.0 g of apples, pears, and tomatoes, respectively, were transferred into a 25 mL Erlenmeyer flask, and 10.0 mL acidified methanol was added. The flask was then partially immersed into a KQ 500DB ultrasonic cleaning bath (Kunshan Ultrasonic, Jiangsu, China) adjusted at 200 W. The bottom of the flask was about 5 cm above the bottom of the bath. The solvent surface in the flask was kept at the level of water in the ultrasonic bath. Water in the ultrasonic bath was circulated, and regulated at a constant temperature to avoid a temperature rise caused by ultrasonic exposure. After a set time (5.0 min), the extract was transferred into a 25 mL centrifuge tube, and centrifuged for 10 min at 7,800 rpm. The ultrasound assisted extraction was carried out again. The supernatant was collected and combined into a 25 mL volumetric flask, and then methanol added to dilute up to graduation. Finally, this solution was filtered through a 0.45 μm nylon syringe filter (Agilent Technologies, Germany) before injecting it into the chromatographic system.

The conventional extraction method by stirring with a mixer was used as a reference for comparison with the ultrasound extraction method. Apples,

pears, and tomatoes (5.0 g) were extracted with 25 mL of acidified methanol by stirring with a mixer (150 rpm) for 10, 30, and 60 min, respectively, at 25°C. Then, the extract was processed in the same manner as ultrasound extraction.

HPLC-APCI-MS Analysis

Separations were performed on a Zorbax SB C_{18} column (150 mm × 2.1 mm i.d., 5 μm particle size, Agilent Technologies, USA) using 0.1% acetic acid/methanol (25:75, v/v) as mobile phase in isocratic mobile phase at a constant flow rate of 0.50 mL/min. The column was maintained at a constant temperature of 35°C and the injection volume was 20.0 μL. Detection was carried out on an Agilent 1100 series LC/MSD Trap SL mass spectrometer in the positive mode with a full scan mass spectra over the m/z range 50 ∼ 300, using a cycle time of 1 s, a corona current of 4.0 μA, a capillary voltage of 2.8 kV, a capillary exit voltage of −145 V, a dry temperature of 325C, a vaporizer temperature of 425°C, a high purity nitrogen (99.999%) dry gas of 8.0 L/min, a nitrogen nebulizer pressure of 35.0 psi, and a dwell time of 200 ms. The APCI interface and mass spectrometer parameters were optimized by direct infusion of the standard solution (1.0 mg/L) to obtain maximum sensitivity. 6-BAP was detected with APCI in selected ion monitoring (SIM) mode. The quantitative ion was selected by its quasi-molecular ion, m/z $[M + H]^+$ 226.1 for 6-BAP. The SIM peak areas were integrated for quantification.

RESULTS AND DISCUSSION

Detection and Chromatographic Conditions

Preliminary experiments were performed to decide between ESI and APCI interfaces and between positive ion and negative ion modes. There were two main obtained ions m/z 226.1 $[M + H]^+$ and m/z 248.1 $[M + Na]^+$ for ESI in positive ion mode, however, only one, m/z 226.1 $[M + H]^+$ for APCI in positive ion mode and only one, m/z 224.1 $[M\text{-}H]^-$ for both ESI and APCI in negative ion mode. In addition, their signal intensities were significantly differential. Figure 2 illustrates the chromatographic response by both interfaces in the SIM mode. This indicates that the sensitivity by APCI in positive mode is better than that by APCI in negative mode and by ESI in both positive and negative modes. The ESI spectrum generally contained adducts ions with sodium, of which relative abundance were variable and dependent on the working conditions and sample composition for 6-BAP, thus affecting the sensitivity in SIM mode. On the contrary, APCI did not form sodium adducts for 6-BAP since ionization occurs in the vapor phase,

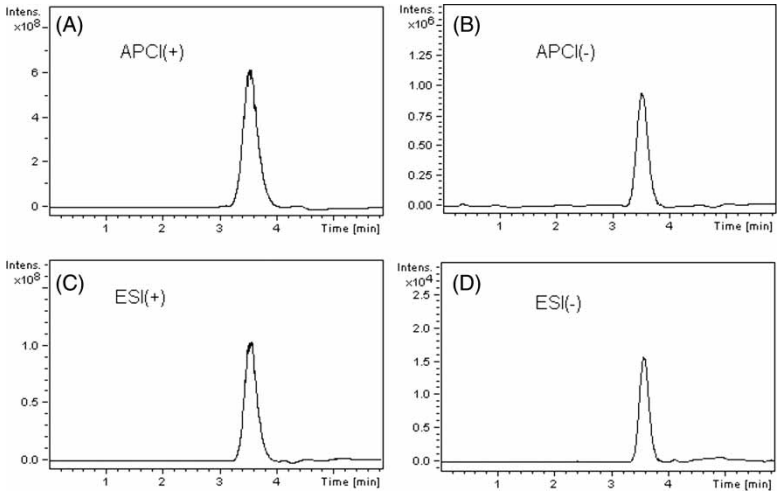

Figure 2. Typical SIM chromatograms of 6-BAP at a concentration of 1.0 mg/L: (A) APCI in the positive mode, (B) APCI in the negative mode, (C) ESI in the positive mode, (D) ESI in the negative mode.

as shown in Figure 3. APCI in positive mode was more robust and gave reproducible spectra of 6-BAP without adduct ion formation. The APCI interface was chosen for the quantitative determination of 6-BAP in apples, pears, and tomatoes owing to its higher sensitivity and selectivity. The criteria for confirming the identity of 6-BAP, which consists of selecting three characteristic ion fragments, is not always met because APCI is a soft ionization technique that provides few fragment ions. However, the correspondence of retention time and molecular weight could provide sufficient specificity for identification of 6-BAP. Another option to improve the selectivity is to perform two analyses for 6-BAP by APCI in both negative and positive modes, in order to obtain an unequivocal confirmation of the identity of 6-BAP.

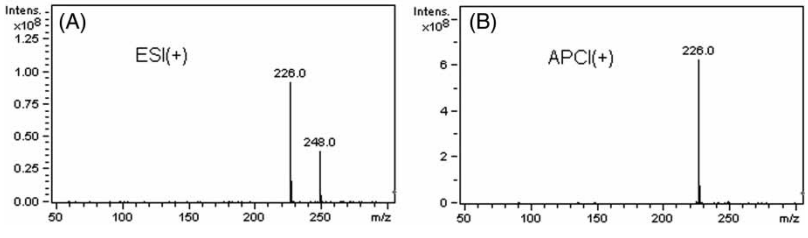

Figure 3. MS spectrum of 6-BAP: (A) ESI in the positive mode, (B) APCI in the positive mode.

The chromatographic conditions were optimized to obtain chromatograms with a good resolution of adjacent interferential peaks within a short analysis time. Different types of chromatographic columns such as the Zorbax SB-C18 column, Hypersil ODS column, and Zorbax XDB column were tested. The Zorbax SB-C18 column proved to be better than the others. Considering the interference of the sample matrix and the simplicity of the analysis, the isocratic elution was used to achieve better separation. After carefully optimizing the composition of the mobile phase, including the ratio of methanol to water and the acetic acid concentration in the mobile phase, 6-BAP was better separated with the matrix. Considering the separation, the run time, and the sensitivity, 0.1 % acetic acid/methanol (25:75, v/v) was used in this experiment.

Optimization of Extraction Conditions

The extraction conditions, including the extraction method, extraction solvent, and extraction time were tested. The results showed ultrasonic extraction with acidified methanol aqueous solution was a preferred method. At the same time, with the concentration of acetic acid in methanol growing, the extraction rates of 6-BAP increased rapidly, while the extraction ratio of 6-BAP had no obvious variety. The extract with low concentration of acetic acid had low viscosity and was easy to separate, while the high concentration of acetic acid had high viscosity and was difficult to separate because of more matrixes dissolving out into the extract. Thereby, ultrasonic extraction with 0.1% acetic acid in methanol for 5.0 min was chosen.

The extractability of 6-BAP was improved by ultrasound extraction, but it was dependent on the solvent employed. Table 1 shows the HPLC-APCI-MS results of the recovery extracted from three spiked samples (5.0, 20.0, and 100.0 ng/g) by different solvents at 25°C. It indicates that the recovery for 5.0 min by ultrasound extraction of 6-BAP was higher than that for 20 min; even equal to the recovery for 60 min by the conventional method, when we used acidified methanol as the extraction solvent. In addition, it also indicates that acidified methanol was the best extraction solvent for extraction of 6-BAP fruits; the recovery by acidified methanol was obviously higher than that by methanol and ethyl acetate. For analytical purposes that were required to rapidly prepare many small volume samples, ultrasound extraction might be more desirable than that by the conventional extraction method.

Calibration and the Limit of Quantification

Calibration and linearity for 6-BAP were performed using a sequence of standard solutions in the concentration range of $0.01 \sim 1.00$ mg/L. Three replicate injections of standards at each concentration were determined. The

Table 1. The recovery (%) of three extraction solvent extracted from three spiked samples by ultrasound extraction and conventional extraction method[a]

	Ultrasound extraction (25ºC)			Conventional method (25ºC)		
Solvent	5.0 min	20.0 min	60.0 min	10.0 min	20.0 min	60.0 min
Spiked at 5.0 ng/g						
Acidified methanol	85.2	86.9	84.9	64.3	80.4	86.6
Ethyl acetate	56.4	58.7	55.7	38.8	42.6	54.8
Methanol	53.2	64.6	67.5	44.4	57.4	68.7
Spiked at 20.0 ng/g						
Acidified methanol	83.6	87.5	88.7	53.6	79.7	87.6
Ethyl acetate	55.7	58.5	59.5	38.9	49.8	56.5
Methanol	59.5	63.3	65.6	43.4	59.5	63.9
Spiked at 100.0 ng/g						
Acidified methanol	89.4	91.2	92.2	61.5	73.7	88.3
Ethyl acetate	58.5	59.4	63.1	43.5	52.4	56.9
Methanol	55.7	62.5	64.6	44.6	53.4	60.9

[a]Each value is the mean of five replicates with a standard deviation no more than 10% of the mean.

response was linear throughout the concentration range of the study, tested with the coefficient of determination (r^2) greater than 0.997. The peak area (X) was then used in conjunction with the calibration curve to derive the concentration (C, ng/g) in fruits. The correlation equation was $C = 4.037 \times 10^{-6} X - 3.253 \times 10^{-4}$ for 6-BAP.

The limit of quantification (LOQ) for 6-BAP was determined using uncontaminated apples, pears, and tomato samples spiked at a low of 5.0 ng/g, extracted with acidified methanol, detected in SIM mode, and evaluated by the criterion that the signal to noise ratio (S/N) should be >10, for quantitative purposes. The LOQ was 2.0 ng/g for 6-BAP.

Accuracy and Precision

Recovery of 6-BAP was determined by spiking at three different concentrations (5.0, 20.0, and 100.0 ng/g), which was treated according to the procedure described above. The recoveries were 89.0%, 94.1%, and 84.7%, respectively, as shown in Table 2.

The intra-day precision was evaluated by performing five replicates of three spiked samples (5.0, 20.0, and 100.0 ng/g) including extraction

Table 2. The recoveries and the precisions of the method for spiked samples

Analyte	Added (ng/g)	Found (ng/g)[a]	Recovery (%)	RSD (%)	
				Intra-day[b]	Inter-day[c]
6-BA	5.0	4.45 ± 0.28	89.0	6.3	7.6
	20.0	18.81 ± 0.96	94.1	5.1	6.5
	100.0	84.68 ± 4.19	84.7	5.0	8.5

[a]Determined in one day.
[b]n = 5.
[c]n = 5 replicates × 3 days within a 7-day period.

procedures. The inter-day precision was also evaluated by performing five replicates of three spiked samples (5.0, 20.0, and 100.0 ng/g), including extraction procedures each day on three different days within a 7 day period. The results of the intra- and inter-day precision (RSD) on the basis of peak area were less than 6.3% and 8.5%, respectively, which are listed in Table 2, indicated that the accuracy and precision of the proposed method are sufficient for the determination of 6-BAP in apples, pears, and tomatoes.

Application to the Real Samples

The viability of HPLC–APCI–MS to determine 6-BAP residue in fruits was evaluated by analyzing ten samples of apples, pears, and tomatoes, respectively. The results show that six samples of apples contained 6-BAP residue, which ranged from $5.8 \sim 150.3$ ng/g; four samples of pears ranged from $12.3 \sim 74.5$ ng/g, seven samples of tomatoes ranged from $54.5 \sim 1243.4$ ng/g, and the others were less than the LOQ of 6-BAP. The data indicate that 6-BAP was used in the course of most fruit production.

CONCLUSIONS

An analytical method of 6-BAP in apples, pears, and tomato samples with ultrasound extraction and LC–APCI–MS was developed. The direct injection method is easy to perform with no sample pretreatment steps. Meeting all the requirements for the validation of an analytical methodology, the inter- and intra-day accuracy and precision data were less than 10%. It can be used for the monitoring of 6-BAP residue in fruit samples because of its simple sample preparation and lower LOQ.

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