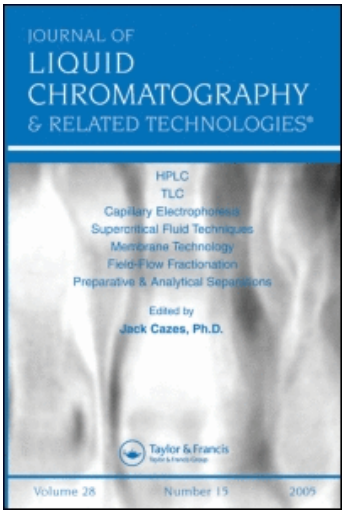

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Determination of 2-Keto Acids and Amino Acids in Plant Extracts

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DETERMINATION OF 2-KETO ACIDS AND AMINO ACIDS IN PLANT EXTRACTS

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ABSTRACT

2-keto acids and amino acids were extracted using liquid nitrogen and 0.25 N HCl. The keto acids and amino acids were seperated by cation exchange chromatography on AG50W-X8 resin. The cation exchange chromatography is vital for the determination of keto acids. The keto acids were derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB), a specific derivatizing agent for 2-keto acids. The derivatized keto acids were quantified by reversed phase high performance liquid chromatography (HPLC). This assay is highly sensitive and can measure as low as 10 fmole of the keto acids per 10 µl injection. The amino acids were analyzed by an automatic amino acid analyzer. These methods were used to show that 2-ketobutyrate (2-KB) and 2-aminobutyrate (2-AB) accumulate in plants treated with an acetohydroxyacid synthase inhibiting herbicide.

INTRODUCTION

Imidazolinones, sulfonylureas and triazolopyrimidines kill plants by inhibiting acetohydroxyacid synthase, a key enzyme leading to the biosynthesis of valine, leucine and isoleucine (1). This enzyme condenses two moles of pyruvate to produce acetolactate or a mole of pyruvate and a mole of 2-KB to produce acetohydroxybutyrate. It has been suggested that inhibition of AHAS by sulfonylureas leads to accumulation of 2KB which is toxic to microbes (2-

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5). Based on this finding, it has been speculated that AHAS inhibitors kill plants due to the accumulation of 2KB, the reaction product of threonine dehydratase. Due to this reason we have been interested in quantitation of 2-KB in plants.

There are several published methods for the determination of 2-KB and other 2-keto acids in plasma and urine (6-10). However, there is no sensitive assay for determination of 2-KB in plant extracts. Derivatizing agents such as dihydrophenylhydrazine, o-phenylenediamine and 1,2-diamino-4,5-methylenedioxybenzene have been used for spectrophotometric or fluorometric determinations of 2-keto acids. Several published methods used for quantitation of keto acid in plasma or urine (6-10) were attempted, however, none of them were successful because the concentration of keto acids in plant extracts is very small and the spiked keto acids were lost when mixed with the plant extracts. Here, we report a method of extraction and derivatization that has been successfully used for the determination of 2-keto acids in various plant extracts. Furthermore, the same extraction method allowed preparation of samples for amino acid analysis.

MATERIALS AND METHODS

Plant Material

Plants were grown in a growth chamber at 30/20°C day/night temperature and 16 h daylength. Maize seeds were germinated on a wet paper toweling and then five-day old seedlings were transferred to 50 ml plastic tubes covered with aluminum foil to eliminate light and containing 35 ml of a complete nutrient solution (11) which was changed daily. Plants were treated with 10 μM imazaquin when the fourth leaf began to emerge from the whorl. The leaf sheath/shoot meristem region was extracted to measure the effects of treatments on keto acids and amino acids. At least two replications with 2-5 plants per replication were used for various measurements. The experiments were conducted several times, however, the data for one example of each representative experiment is presented.

Extraction of keto acids and amino acids

The plant material was pulverized in liquid nitrogen and then further ground in 0.25N HCl containing 0.1 mg/ml 2-oxopentanoate (internal standard for keto acid analysis) and 500 nmol/ml L-α-amino-β-guanadinopropionic acid (internal standard for amino acid analysis). Two ml of extraction solution was used for each g of tissue fresh weight. The extract was centrifuged at 25,000 g for 15 min. An aliquot of the supernatant (0.25 ml) was loaded on a cation exchange column (AG 50W-X8 from Bio Rad, Richmond, CA; resin bed volume=4ml) pre-equilibrated with 0.01 N HCl. The column was washed with 1.5 ml of 0.01 N HCl and then the keto acids were eluted in 2 ml of 0.01 N HCl. Amino acids bind with this resin and were eluted with 4 x 4 ml aliquots of 9N ammonium hydroxide.

Derivatization of keto acids

The method of derivatizing the keto acids to form quinoxalones was adapted from Nakamura et al. (9) and Wang et al. (10). A 5 mM solution of DMB was prepared in a fresh solution of 1.5 N HCl containing 20 mM sodium dithionite and 1 mM β-mercaptoethanol. A 250 µl aliquot of the solution containing the keto acids was mixed with an equal aliquot of DMB solution. The mixture was vortexed and then heated in a boiling water bath for 45 min. The derivatized keto acids solution was diluted in the HPLC running buffer for analysis.

HPLC of keto acids

The HPLC conditions are identical to those described previously (9). The HPLC system consisted of a Beckman 112 solvent delivery module (Beckman, Fullerton, CA), DYNAMAX Model FL-1 fluorescence detector (Rainin, Woburn, MA), a WISP 710B automatic sampler and a Waters 840 data integration system (Waters Assoc., Milford, MA). A Radial-PAK cartridge C_{18} reversed-phase column (5-µm particle size; 100 x 8 mm i.d.) was used which was connected with a stainless steel guard column packed with C_{18} resin. The mobile phase (acetonitrile-methanol-40 mM phosphate, pH 7, 12/13/25, v/v/v) was run at a flow rate of 1.5 ml/min. For the fluorometric analysis, the excitation and emission wavelengths were 367 and 446 nm, respectively.

Amino acid analysis

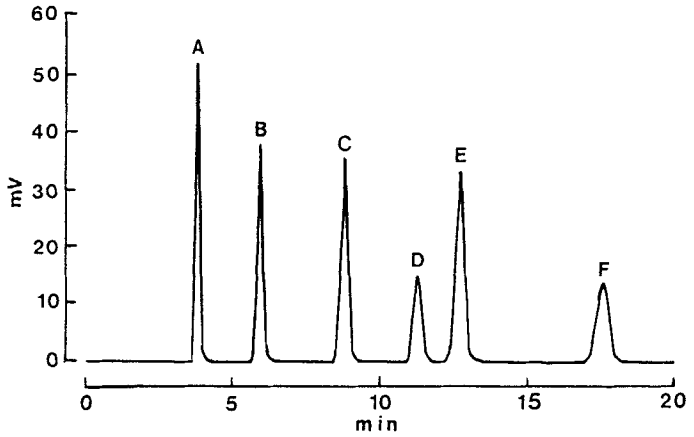
Amino acids eluted from the cation exchange column were freeze dried and then dissolved in Na-S buffer (Beckman, Fullerton, CA). The solution was filtered to remove the particulate matter and the amino acid composition was determined on a Beckman 7300 amino acid analyzer.

RESULTS AND DISCUSSION

o-Phenylenediamine was used for determination of keto acids in preliminary experiments, however, almost all of the keto acids spiked in the plant extracts were lost during the derivatization process. Once the extraction and derivatization conditions were optimized, the sensitivity of detection using *o*-phenylenediamine was not high enough to detect 2-KB in plant extracts. It has been reported that DMB is a specific fluorogenic reagent for 2-keto acids (9). DMB was shown to be the best precolumn derivatization reagent, in terms of sensitivity and reactivity, in the HPLC evaluation of eight 1,2-diaminobenzene derivatives. Due to this reason, DMB was chosen and used in our subsequent experiments.

Several different extraction conditions were evaluated. Pulverizing plant material using liquid nitrogen allowed rapid cell breakage, preparation of the sample in powder form, and reduced enzymatic attack on the compounds of interest. This step is especially important for plant material containing high polyphenol oxidase activity which makes the sample dark brown in a very short time and thereby interferes with analyses. Further grinding the sample in strong acidic conditions (0.25N HCl) denatures proteins and thereby reduces the risk of enzymatic attack on keto acids and amino acids.

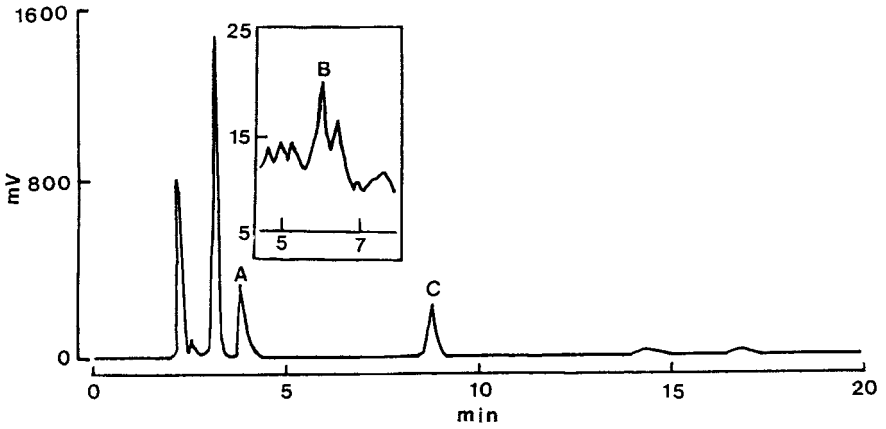
There was poor recovery of keto acids spiked in the crude extracts of plants prepared as described above. Apparently, there was some interference from the metabolites present in the plant extracts. It was found that cation exchange chromatography on AG 50W-X8 (Bio Rad, Richmond, CA) removed the interfering compound(s). The keto acids do not bind with the resin and are collected in the passthrough. Amino acids present in the extract bind with the resin which were eluted with 9N ammonium hydroxide. Routinely, greater than 90% of the spiked keto acid and amino acid were recovered using this extraction procedure.



1. HPLC of authentic pyruvate (peak A), 2-KB (peak B), 2-ketopentanoate (peak C), 2-ketoisovalerate (peak D), 2-ketoisocaproate (peak E) and 2-ketomethylvalerate (peak F) following derivatization with DMB.

The conditions for derivatization with DMB and HPLC are almost identical wth those described previously (9, 10). Figure 1 shows a typical chromatogram obtained with a standard mixture of 2- keto acids. Each of these keto acids gave single peaks with identical retention times when they were subjected to the same procedures individually. 2-ketopentanoate was not seen in the plant extracts in the preliminary experiments, therefore, this keto acid was used as an internal standard in all subsequent experiments. Using this protocol, as low as 10 fmol of a keto acid per 10 µl injection was detected. The extraction and derivatization procedure described here worked for all samples that we examined. These included samples from tissue culture (Black Mexican Sweet corn cells), dicots (cocklebur, lima bean, and sunflower) and monocot (corn). In this paper we have presented results obtained only from corn.

Several peaks of different keto acids were detected in the extracts of corn shoots (Fig. 2). In addition to the retention times of the standards, spiking the samples with different keto acids led to the identification of various keto acids. Of all keto acids quantified, pyruvate was present



2. HPLC of DMB-derivatized keto acids in extracts of corn seedlings. The inset shows the enlarged view of the chromatogram between 5 and 7 min where 2-KB (Peak B) elutes. Pyruvate (Peak A) and 2-ketopentanoate (internal standard; Peak C) are shown in the main chromatogram.

in the highest concentration (Table 1). Only trace amounts of other keto acids of the branched chain amino acid biosynthetic pathway were present in corn shoot extracts. Some other keto acids were present in high concentration (Fig. 2), however, We made no attempts to identify these keto acids.

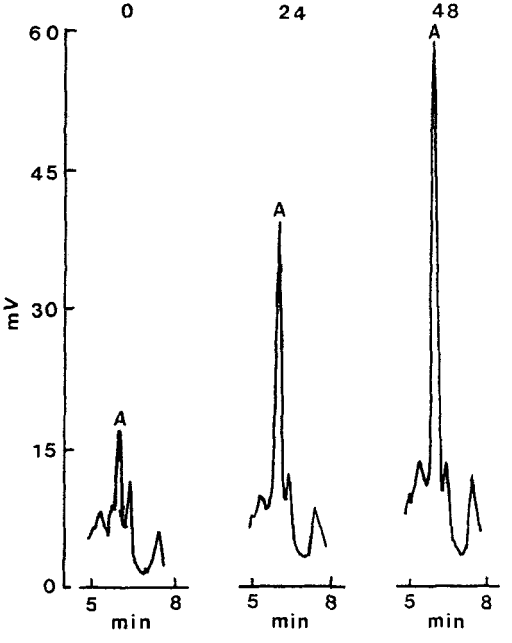
Imazaquin at 10 μM caused accumulation of 2-KB in corn shoots (Fig. 3, Table 2). This result is consistent with the previously reported accumulation of 2-KB in *Salmonella* grown in the presence of an AHAS inhibiting herbicide (2-5). A progressive increase in accumulation of 2-KB over time indicates that imazaquin prevents utilization of 2-KB by AHAS. Since the pathway before AHAS is unaffected, carbon continues to flow in the aspartate pathway leading to the accumulation of 2-KB.

Amino acids eluted from AG 50W-X8 resin were freeze dried and then dissolved in Na-S buffer and analyzed on Beckman 7300 amino acid analyzer. Excellent recovery of amino acids

Table 1

Levels of different keto acids of the branched chain amino acid biosynthetic pathway in corn seedling.

Keto acid	nmoles / g fresh weight
Pyruvate	85.0
2-ketobutyrate	0.2
2-ketoisovalerate	0.2
2-ketoisocaproate	< 0.05
2-ketomethylvalerate	< 0.05



3. Accumulation of 2-KB (Peak A) in corn seedlings treated with 10 μM imazaquin at 0, 24 and 48 h.

Table 2

Accumulation of 2-KB and 2-AB in corn seedlings treated with 10 μM imazaquin.

Time (h)	2-KB	2-AB
	(nmoles / g fresh weight)	
0	0.2	1
24	4.8	384
48	10.3	1269

was obtained in plant samples prespared in this way (chromatogram and data not shown). Corn shoots contain very low levels of 2-AB (Table 2). However, imazaquin treatment caused accumulation of 2-AB which increased progressively with time. Interestingly, the levels of 2-AB were about 100-fold higher than the levels of 2-KB. Since 2-AB is a transamination product of 2-KB, our observation suggests that the two compounds are in equilibrium in vivo and the equilibrium is in favor of 2-AB.

In summary, a method of simultaneous extraction of 2-keto acids and amino acids has been developed. The keto acids and amino acids are separated by cation exchange chromatography. This chromatography is also vital for the determination of keto acids. The keto acids are derivatized with DMB and then analyzed by reversed phase HPLC. The amino acids are quantitated by an amino acid analyzer. These methods were used to demonstrate that 2-KB and 2-AB accumulate in imazaquin treated corn shoots.

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