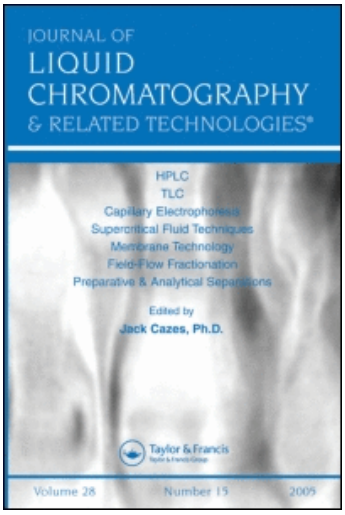

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QUANTIFICATION OF CREATINE IN NUTRITION SUPPLEMENTS BY THIN LAYER CHROMATOGRAPHY-DENSITOMETRY WITH THERMOCHEMICAL ACTIVATION OF FLUORESCENCE QUENCHING

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ABSTRACT

A quantitative method using silica gel HPTLC plates with fluorescent indicator; thermochemical, reagent-free reaction to produce fluorescence quenched zones; and automated UV absorption densitometry has been developed for the determination of creatine in nutrition supplements. Eight products containing different amounts of creatine monohydrate and additional ingredients were analyzed. Accuracy was validated by analysis of a certified reference sample and precision by performing replicated analyses.

Accuracy was found to be within 0.2% of the certified value, and precision was 3-4% relative standard deviation.

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INTRODUCTION

Creatine is widely used by athletes, body builders, and weight lifters as a nutritional supplement for increased power, muscle development, and faster recovery from exercise. The preferred form used as a dietary supplement is the white crystalline creatine monohydrate, which is sold "pure" or mixed with other ingredients such as vitamins, minerals, amino acids, and flavorings.

Thermochemical, reagent-free conversion of creatine to a fluorescent derivative on amino-bonded silica gel plates(1) has been used for determination in human urine(2) and other biological samples.(3) Additional published methods for creatine TLC analysis, located by a computer-assisted search of Chemical Abstracts, include separation of creatine phosphate from other naturally occurring phosphorus compounds on cellulose and polyethyleneimine-cellulose layers;(4) detection on silica gel plates by an improved N-chlorination procedure;(5) separation of creatine from arginine in biological fluids on silica gel plates with detection as a red zone using Sakaguchi reagent;(6) and separation of 13 guanidino compounds including creatine by TLC on silica gel,(7) cellulose,(7) and ion-exchange layers.(8)

We describe, here, a high performance thin layer chromatography (HPTLC) method for determination of creatine that is based on formation of zones that quench fluorescence by heating a silica gel plate containing a phosphor. It is the first paper that reports a quantitative TLC method for analysis of creatine in nutritional supplements. The method is more selective than the standard perchloric acid titration method for assay of creatine. It would have advantages over high performance column liquid chromatography (HPLC) because it is simpler to perform, and the ability to analyze multiple samples against standards chromatographed on the same plate leads to increased accuracy, speed, and sample throughput and lower cost and solvent usage per sample.

The use HPLC has been reported for determination of creatine in rat brain and eye,(9) neocortical slices and cell cultures,(10) serum,(11,12) and fish tissue(13) and creatine phosphate in mammalian skeletal muscle.(14) No HPLC method for assay of creatine in nutrition products was found by computer search.

EXPERIMENTAL

Preparation of Standard and Sample Solutions

Anhydrous creatine (N-amidinosarcosine; #C0780, Sigma, St. Louis, MO, USA; CAS registry no. 57-00-1; 100% purity) standard solution was prepared at a concentration of 1.00 mg/mL in deionized water. Products containing creatine monohydrate were purchased from a health-food store or pharmacy, and appropriate weights (0.100-1.50 g/100 mL) were dissolved in water to produce sample solutions of 1.00 mg/mL, based on label values (see Table 1). All products were

powders except number 5, which was in the form of capsules. Solutions of samples 5, 6, and 8 (Table 1) were magnetically stirred for 10 min to ensure that creatine monohydrate was completely dissolved, and the undissolved material was allowed to settle for 15 min prior to analysis.

For use in validation of the method, a standard of creatine monohydrate (#C3630, Sigma, CAS registry no. 6020-87-7) was purchased and dissolved in water at a concentration of 1.00 mg/mL; this standard was received with a certificate of analysis stating that it contained 10.8% water as determined by Karl Fischer titration, or 89.2% creatine on an anhydrous basis. The standard and all samples were prepared in 100 mL volumetric flasks.

Thin Layer Chromatography

Analyses were performed on 20 x 10 cm Whatman (Clifton, NJ, USA) LHPKDF high performance silica gel plates with fluorescent indicator, preadsorbent zone, and 19 channels (No. 4806-711). The plates were precleaned by development to the top with dichloromethane-methanol (1:1) and dried before use.

Table 1. Analyses of Creatine Monohydrate Nutrition Supplements

Product	% Creatine Found	Label Value(a)	Other Ingredients
1	93.6	100	none
2	93.6	100	none
3	90.7	100	none
4	11.5	12.2	(b)
5	71.0	66.7(c)	(d)
6	8.74	9.84	(e)
7	96.5	100	(f)
8	17.2	20.8	(g)

(a) % Creatine monohydrate.
(b) Dextrose, taurine, flavor, citric acid, beet powder, magnesium phosphate, disodium phosphate, potassium phosphate.
(c) Percentage based on an average weight of 1.24 g of powdered sample/capsule; 5000 mg/6 capsules was stated on the label.
(d) L-glutamine, taurine, gelatin, purified water, MCT, magnesium stearate, silica.
(e) Glucose, glutamine peptide, orange flavor, citric and malic acids, dl-alpha-tocopherol acetate, niacinamide, D-calcium pantothenate, pyridoxine hydrochloride, riboflavin, thiamine mononitrate, folic acid, biotin, chromium picolinate, L-selenomethionine, cyanocobalamin, alpha-lipoic acid, beta-carotene, beet juice, xanthan gum.
(f) Despite stating 5000 mg of creatine/5 g of powder, silica was listed as an ingredient.
(g) Glucose, calcium-beta-hydroxy beta-methyl butyrate monohydrate, trimethylglycine, orange flavor, beta-carotene, beet juice, xanthan gum, aspartame.

Standard and sample solutions were applied to the preadsorbent areas of adjacent channels using a 10 µL Drummond (Broomall, PA, USA) digital microdispenser. The volumes applied for each analysis were 1.00 µL, duplicate 2.00 µL, and 4.00 µL of the standard (1.00-4.00 µg creatine) and duplicate 2.00 µL of the sample solutions (ca. 2.00 µg theoretical content). The initial zones were dried with a hair dryer on high heat setting for ca. 3 min before development.

Plates were developed to a distance 6 cm beyond the origin (preadsorbent-silica gel interface) with the mobile phase consisting of acetonitrile-deionized water (7:3) in a vapor-equilibrated Camag (Wilmington, NC, USA) twin-trough chamber containing a saturation pad (Analtech, Newark, DE, USA). The development time was ca. 15 min.

After development, the mobile phase was evaporated from the plate by drying in a fume hood for 10 min with a hair dryer, and it was then heated for 5 min at 160°C on a Camag plate heater. The resultant fluorescence-quenched zones of creatine standards and samples were measured by linear scanning at 254 nm by use of a Camag TLC Scanner II with deuterium source, slit length 4, slit width 4, and scanning rate 4.0 mm s^{-1}.

The CATS-3 software controlling the densitometer produced a calibration curve by linear regression of the weights and areas of the standard zone scans and interpolated the weights of the sample zones from their scan areas. For each analysis, the percentage creatine was calculated by multiplying the average weight of creatine in the 2.00 µL sample aliquots times the factor (100×10^3 µL/2.00 µL), dividing by the µg of sample weighed into the 100 mL volumetric flask, and multiplying the quotient by 100.

Accuracy of the new method was validated by analysis of the certified creatine standard. Precision was validated by spotting four 2.00 µL aliquots of several samples and calculating the relative standard deviations of the scan areas. Further evaluation of reproducibility was obtained by calculating the percent difference between the scan areas of the duplicate sample and standard zones spotted in each analysis.

RESULTS AND DISCUSSION

Manual spotting of samples and standards was employed because the lack of creatine solubility in volatile, organic solvents precluded the use of our spray-on Camag Linomat automated applicator. Application of 1.00-4.00 µL volumes of water solutions as single spots with a 10 µL Drummond digital microdispenser onto preadsorbent plates was found to be the optimum method.

After the heating step, creatine was detected as a compact, dark band against a bright green background at R_f 0.30 when viewed under 254 nm UV light. Despite the presence of many additional ingredients in some of the samples

(Table 1), only one additional zone was detected in chromatograms of samples 4, 6, and 8. It appeared at R_f 0.51 as an orange zone in visible light and as a dark zone under UV light after heating, and it was well separated from creatine and did not interfere with quantification by scanning. Zones of creatine were not visible under UV light before thermochemical activation. We were unable to produce fluorescent zones of creatine, as reported in the literature,(1-3) by heating on Merck NH_2F_{254} HPTLC plates at temperatures ranging from 135-170°C for 3-12 min. Fluorscence quenched zones were formed by heating on the Whatman HPTLC silica gel plates specified above and on Whatman LK5DF TLC silica gel plates or Merck $60CF_{254}$ HPTLC silica gel plates. The Whatman HPTLC plates provided the optimum detection sensitivity and reproducibility for thermochemical reaction of creatine.

Table 1 shows the percentage creatine found in the eight commercial creatine nutrition supplements we analyzed by the new TLC method, along with their label values, stated as creatine monohydrate content, and additional ingredients present. A sample that is 100% creatine monohydrate has a theoretical creatine content of 87.9%, as calculated by dividing the formula weight of creatine (131) by the formula weight of creatine monohydrate (149) and multiplying by 100. Pure creatine monohydrate products that lose some water content on storage will assay above this creatine value while those that might gain water will give a lower value. Products with other ingredients may assay higher or lower than theoretical, depending on the time and conditions of storage and content tolerances allowed by each manufacturer. Experimental weight percentage values ranged from 8.74-96.5% of creatine, and all experimental values were higher than the theoretical amounts except for product 8.

Accuracy was validated by analyzing a creatine monohydrate sample certified to contain 89.2% creatine. Our analysis yielded an average value of 89.0% creatine, representing 99.8% recovery or 0.22% error. Products 1 and 2, which were the same brand purchased in different stores on dates several months apart, both assayed at 93.6%, giving further evidence of the accuracy of the method.

Reproducibility was determined by spotting four 2.00 μL of this certified sample and of sample 7 (Table 1). The relative standard deviations of the scan areas were 3.5% and 4.3%, respectively. Percent difference values of the areas of duplicate samples and standards spotted in the analyses averaged 3.7% with a range of 0.76-7.4%. Linearity (R) values of the calibration curves for the four standards spotted on each plate ranged from 0.975-0.996, with a mean of 0.989.

It has been demonstrated above that accuracy and precision of the new method meet the guidelines set by the International Conference on Harmonization (ICH) for assay of pharmaceutical products(15) and are similar to values reported regularly in the literature for HPTLC and HPLC analysis of pharmaceutical products and foods. For example, Renger(16) reported accuracy (recovery) of 101.3% (HPLC) and 98.5-102.8% (HPTLC) and repeatability or precision

(RSD) of 0.9-5% (HPLC) and 1.2-2.8% (HPTLC) in a comparative study of pharmaceutical analysis. The method will be valuable for use by manufacturing companies in qualitative or quantitative quality assurance analysis and by government laboratories for surveying products being sold.

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