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Levels of creatine, organic contaminants and heavy metals in creatine dietary supplements

Sabrina Moret [a,*], Annalisa Prevarin [a], Franco Tubaro [b]

[a] Department of Food Science ,Via Sondrio 2A, University of Udine, 33100 Udine, Italy
[b] Department of Chemical Science and Technology, Via Cotonificio 108, University of Udine, 33100 Udine, Italy

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ABSTRACT

High performance liquid chromatography (HPLC) has been optimised for the analysis of the creatine content and possible organic contaminants in 33 samples of creatine supplements from the market. Creatinine resulted to be the major organic contaminant (44% of the samples over 100 mg/kg). About 15% of the samples had dihydro-1,3,5-triazine concentrations exceeding the detection limit of 4.5 mg/kg (maximum 8.0 mg/kg) and a dicyandiamide concentration over 50 mg/kg, while none of the samples were contaminated with thiourea. The heavy metals (arsenic, cadmium, mercury and lead) content was also assessed by means of inductively coupled plasma mass spectrometry (ICP-MS). Only mercury was present in detectable amounts (at levels lower than 1 mg/kg).

1. Introduction

Creatine is an amino acid derivative with its highest amount in the skeletal muscle. It is synthesised endogenously from arginine, glycine and S-methylmethionine, but can be also supplied through the diet, mainly from meat and fish (Harris, 2001). Since 1993, when it was introduced for the first time as a dietary supplement, it became very popular among sports people and as therapeutic agent (Benzi & Ceci, 2001; Persky & Brazeau, 2001; Wyss & Kaddurah-Daouk, 2000). Several studies have proved the positive benefits of creatine supplements, used in an appropriate dosage, in improving exercise performance (in events requiring high energy activity, especially of a repeated nature), reducing fatigue, accelerating both energy recovery and muscle growth, increasing muscle strength, promoting muscle size without affecting body fat (Flisinska-Bojanowska, 1996; Fry & Morales, 1995; Poortmans & Francaux, 2000; SCF, 2000). Nevertheless, other researchers have shown that an excessive intake of creatine is associated with gastrointestinal distress, diarrhoea, muscle cramps and renal dysfunctions (Benzi, 2000; Brudnak, 2004; Clark, 1997; Tarnopolsky & Martin, 1999).

Creatine supplements are commercially available mainly as creatine monohydrate (CrM). However, other forms such as creatine citrate (CrC), creatine phosphate, creatine piruvate, creatine malate, Kre-Alkalyn® (CrA) (a special form of creatine monohydrate buffered at basic pH) and creatine ethyl ester, are commercially available both in their pure form or in formulation with other ingredients. Currently creatine is prepared by chemical synthesis following two main synthetic routes. The first route, which is more common in the large-scale industrial production, uses as starting materials sodium or potassium sarcosinate and cyanamide (Weiss & Krommer, 1998). Inferior starting materials, non-optimised reaction conditions, as well as inadequate purification (unsufficient washings), results in an increased amount of potential impurities, such as creatinine, dicyandiamide and derivatives of dihydro-1,3,5-triazine (Benzi, 2000; Bizzarini & De Angelis, 2004; EFSA, 2004; Harris, 2001). The alternative low cost route (An, Zheng, & Guoji, 2001), which uses sarcosine (and/or potassium or sodium sarcosinate) and S-methylisothiourea (and/or methylisothiourea sulphate) as starting materials, does not produce dicyandiamide, but can generate thiourea, classified by IARC (1987) as possible carcinogen to humans (group 2B).

Creatinine is a by-product both of creatine metabolism in humans and of creatine industrial production (cyclisation of creatine). Probably the ingestion of creatinine is a safe endeavour but it does not have any ergogenic effects and therefore the maximum admissible amounts need to be defined (Benzi, 2000). Dicyandiamide is a derivative of cyanamide (dimerisation product) probably formed as a result of an incomplete or inefficient chemical process. Even though a detoxification system (able to convert dicyandiamide into the less toxic thiocyanate) is active in many tissues, the acid

* Corresponding author. Tel.: +39 0432 558146; fax: +39 0432 558130.
E-mail address: sabrina.moret@uniud.it (S. Moret).

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environment (stomach acids) may convert dicyandiamide into the more toxic hydrogen cyanide (Benzi, 2000; Brink, 1998; Franco-Obregón, 2007). A tolerable daily intake (TDI) of 1 mg/kg bw per day has been established for dicyandiamide used as a monomer for food packaging material (EFSA, 2004). Dihydrotriazines are isomeric compounds which can be found in creatine products as by-products of non-optimised creatine production. Structurally related compounds are known to be carcinogenic, but specific data concerning dihydrotriazine toxicity in humans are not yet available (Benzi, 2000).

Contamination with heavy metals, whose toxicity is well known, is also possible. The source of contamination can be associated to various causes: the raw materials, the reagents and the solvents used to produce them, the tubing, the equipment and the instrumentation (reaction containers, electrodes, etc.) that can come in contact with the product (e.g. lead is used in metallic alloys), containers where products are stocked or packed (e.g. mercury and cadmium are employed in the plastic industry). According to the EFSA Opinion (2004), creatine monohydrate products should comply with the following specifications: creatinine maximum 100 mg/kg, dicyandiamide maximum 50 mg/kg, dihydro-1,3,5-triazine not detectable (lower than the detection limit of 4.5 mg/kg), heavy metals maximum 10 mg/kg of which arsenic, cadmium, mercury and lead maximum 1 mg/kg. The data on the levels of organic contaminants in creatine supplements are very scarce and mainly refer to creatine monohydrate. Differences in the quality of creatine products from different manufacturers were reported by Brink (1998, 1999), with maximum amounts of 7700 mg/kg for creatinine, 34,000 mg/kg for dicyandiamide and 410 mg/kg for dihydrotriazine. In a survey carried out by the University College Chichester, lower but still significant levels of these contaminants have been found in some products available in the UK and in Europe (Harris, 2001). No data on heavy metal contamination in creatine supplements are available from the literature.

The goal of this research was to carry out a survey on the quality of creatine products commercialised, under different forms, in Italy and produced in different European countries and in the USA. The products were tested to verify purity (percent of creatine monohydrate) and to assess their contamination level with organic contaminants and heavy metals. To achieve this goal an HPLC method based on cation exchange chromatography and UV detection was optimised and applied to analyse organic contaminants, while sample mineralisation (by means of microwave digestion) followed by inductively coupled plasma mass spectrometry (ICP-MS) was applied for the determination of heavy metals.

2. Materials and methods

2.1. Chemicals and standards

The reagents and the standards used for HPLC analysis of creatine and organic contaminant are the following: purified water obtained with a Milli-Q system (Millipore, Bedford, MA, USA), ammonium dihydrogen phosphate ($NH_4H_2PO_4$), phosphoric acid (H_3PO_4) 85% v/v, creatine anhydrous (Fluka, Büchs, Switzerland), creatinine (Fluka), thiourea (Fluka), dicyandiamide (Sigma–Aldrich, Steinheim, Germany), dihydro-1,3,5-triazine (kindly furnished by Manfred Wildenauer from Degussa). The working standard solutions of each organic contaminants (to be used as external standards) were prepared by diluting stock standard solutions with purified water in order to obtain 0.05 μg/l of thiourea, 0.20 μg/l of dicyandiamide, 0.85 μg/l of dihydrotriazine and 0.50 μg/l of creatinine. The standard solution of creatine was prepared by weighting 300 mg of creatine into a 100 ml volumetric flask and taking to volume with purified water. Creatine and creatinine solutions were prepared fresh every day (these are stable for 3 days at 4 °C).

The reagents and the standards used for heavy metals analysis are the following: nitric acid (HNO_3) 65% v/v (Merck Suprapur, Darmstadt, Germany), hydrogen peroxide (H_2O_2) 30% v/v (Merck Suprapur), sodium borohydride ($NaBH_4$) (Fluka); sodium hydroxide (NaOH) (Prolabo, Briare the Canal, France), deionised ultrapure water ($R > 18$ MΩ) obtained with a Elgastat device UHQ-PS (Elga, Buckinghamshire, UK), standard solution of Cd at the concentration of 1000 mg/l in HNO_3 5% v/v (Merck), standard solution of Pb at the concentration of 1000 mg/l in HNO_3 5% v/v (Merck), standard solution of As at the concentration of 1000 mg/l in HNO_3 5% v/v (Merck); standard solution of mercury (Hg) at the concentration of 10,000 mg/l in HNO_3 at 8–10% v/v (Sigma, Milwaukee, USA). All the reagents used were of ultrapure analytical degree.

2.2. Samples

Thirty-three creatine over-the-counter were randomly purchased from the Italian market. Twenty-three samples were based on CrM, six on CrA and three on tri-creatine citrate (CrC). The samples were different in formulation (labelled as pure creatine 100% or in formulation with other ingredients and excipients) and dosage form (powder, micronised powder, capsules and tablets). Table 1 gives a more exhaustive description of the samples analysed. The tablets were previously reduced to powder with a mortar, while the capsules were opened to obtain the powder. At least three tablets or capsules were used to obtain a representative sample.

2.3. Sample preparation

For HPLC analysis of creatine and organic contaminants, a known amount (300 mg) of sample powder was precisely weighed into a 100 ml volumetric flask, dissolved in water with the help of a Branson (model 5200) ultrasonic bath (Energiewieg, Soest, The Netherlands) and made up to the volume with Milli-Q water. In order to limit creatine conversion to creatinine (which is favoured by heating during sonication), sonication times not longer than 2 min were used. Samples were filtered through 0.45 μm syringe filter (Alltech, Milan, Italy) before injection into the HPLC apparatus. For heavy metal determination, 500 mg of sample powder were mineralised in a microwave digester with 3 ml of HNO_3 65% v/v and 0.5 ml of H_2O_3 30% v/v and diluted to 100 ml in a volumetric flask with ultrapure water.

2.4. HPLC analysis of creatine and organic contaminants

HPLC determination of creatine and organic contaminants was performed with a Pro Star solvent delivery pump, model 210 (Varian, Palo Alto, CA, USA), equipped with a manual injector and a 20 μl loop. The column was a Nucleosil 100-5-SA, version Cation Strong, 250 × 4.6 mm i.d., 5 μm of particle size (Bischoff, Leonberg, Germany). The mobile phase was prepared by dissolving 23 g of ammonium dihydrogen phosphate in 1 l of Milli-Q water and adjusting the solution to pH 4.0 with phosphoric acid. For the analysis of CrC samples in formulation with other ingredients able to interfere with analyte detection, the pH was adjusted to 3.8. Before use, the buffer was filtered and degassed. Elution was carried out in the isocratic mode at a flow rate of 1.3 ml/min. Detection was performed with a programmable Pro Star UV detector, model 320 (Varian, Palo Alto, CA, USA). Optimised wavelengths used during elution are the following: 215 nm for thiourea and dicyandiamide (0.0–3.0 min), 230 nm for creatine (3.0–4.0 min), 240 nm for dihydro-1,3,5-triazine (4.0–6.5 min) and 215 nm for creatinine (6.5–9.0 min).

Table 1
Description of the samples.

Sample	Formulation	Dosage form	Provenience
Creatine monohydrate (CrM)			
CrM1	100% CrM	Powder	USA
CrM2	100% CrM	Micronised powder	EU
CrM3	100% CrM	Capsules	EU
CrM4	100% CrM	Powder	
CrM5	100% CrM	Micronised powder	
CrM6	100% CrM	Powder	
CrM7	100% CrM	Powder	EU
CrM8	100% CrM	Powder	USA
CrM9	100% CrM	Powder	USA
CrM10	100% CrM	Micronised powder	USA
CrM11	100% CrM	Micronised powder	EU
CrM12	100% CrM	Micronised powder	
CrM13	100% CrM	Micronised powder	EU
CrM14	100% CrM	Micronised powder	EU
CrM15	100% CrM	Micronised powder	
CrM16	100% CrM	Powder	
CrM17	100% CrM	Powder	EU
CrM18	100% CrM	Micronised powder	
CrM19	100% CrM	Powder	EU
CrM20	77% CrM plus other ingredients[a]	Tablets	EU
CrM21	100% CrM	Powder	
CrM22	CrM (% not declared) plus other ingredients[b]	Effervescent. powder	
CrM23	100% CrM	Micronised powder	EU
Kre-Alkalyn® (CrA)			
CrA1	% not declared	Capsules	USA
CrA2	100% CrA	Capsules	EU
CrA3	% not declared plus other ingredients[c]	Capsules	
CrA4	% not declared plus other ingredients	Capsules	EU
CrA5	100% CrA	Capsules	USA
CrA6	100% CrA	Powder	
Tri-creatine citrate (CrC)			
CrC1	45% CrC plus other ingredients[d]	Effervescent powder	EU
CrC2	31% plus other ingredients[e]	Effervescent powder	EU
CrC3	100% CrC	Effervescent powder	EU

[a] Microcrystalline cellulose, carboxymethyl cellulose, magnesium stearate, silica dioxide.
[b] Mono- and polysaccharides, citric acid, sodium carbonate, silica and flowerings.
[c] Microcrystalline cellulose, magnesium stearate.
[d] Dextrose, L-glutamine, sodium bicarbonate, citric acid, aspartame, acesulfame K, ß-carotene, flowerings.
[e] Dextrose, fructose; sodium bicarbonate, citric acid, aspartame, flowerings.

2.5. ICP-MS analysis of heavy metals

For the mineralisation of the samples a microwave digester mod. MLS-1200 (Milestone, Bergamo, Italy) equipped with tetrafluoroethylene vessels mod. ML-34040 (Milestone), was used. As an internal standard, 100 μg/l of Rhodium were used. All spectrometric measurements were performed by a Spectromass-ICP 2000 mod. MSDIA10B (Spectro Analytical Instruments, Kleve, Germany), equipped with a concentric pneumatic nebuliser type Meinhard. To reduce more the detection limits (DLs), the spectrometric technique was coupled to a hydride generator for the introduction of the sample. The transformation of arsenic and mercury into the correspondent hydrides was obtained by employing a reducing agent (sodium borohydride) in hydrochloric acid at room temperature. Such conversion offers the advantage to diminish matrix effects and to pre-concentrate analytes (Pohl, 2004).

Prior to each analysis, a pre-flush period of 10 s was adopted, during which the apparatus was washed with the sample to be investigated. All analytical data were collected under standard laboratory conditions, i.e., not in a clean-room environment. Results were the mean of 10 determinations.

2.6. Method validation

Standard solutions of thiourea, dicyandiamide, dihydro-1,3,5-triazine and creatinine used for HPLC calibration were prepared by diluting the stock solution of each analyte with Milli-Q water to yield 5 or 6 nominal concentrations over the ranges reported in Table 2. Calibration graphs were constructed for each analyte by plotting the chromatographic areas versus analyte concentration (μg/ml). All researched contaminants showed a good linearity response in the range of concentration tested. Table 2 reports for the range of concentrations tested, the linear regression equations and their coefficients of determination (R^2), as well as DLs expressed as the amount of analyte able to generate a signal twice the background noise.

The analytical repeatability of the HPLC method was assessed by performing six replicate analyses (each one injected twice) of the same sample (CrM16). The coefficients of variation lower than 9.0% were obtained for the analytes present in the sample (creatine 1.6%, dicyandiamide 7.7%, dihydro-1,3,5-triazine 8.5% and creatinine 7.1%). The percent recoveries obtained by adding known amounts of each analyte (at a level one to **five times** the estimated **detection limit**) to a solution of creatine monohydrate were all high (above 93%). In order to ensure reliable results, low- and high-level quality control samples (standard solutions) were prepared and injected every day. Blank analyses were also performed daily in order to confirm the absence of carry-over and/or interfering peaks.

The verification of the linearity of the signal for the analysed elements (75As, 114Cd, 202Hg and 208Pb) has been carried out by calibrating the ICP-MS with the respective standard solutions,

Table 2
Linearity and detection limits of HPLC analysis of organic contaminants and ICP-MS analysis of heavy metals.

Analyte	Concentration range (μg/l)	Regression equation	Coefficient of determination (R^2)	Detection limit (mg/kg)
Thiourea	10–5100	$y = 121572x - 1208.0$	0.999	1.0
Dicyandiamine	10–10200	$y = 141233x + 291.2$	1.000	2.0
Dihydro-1,3,5-triazine	17–3435	$y = 56481x + 925.5$	0.999	4.5
Creatinine	50–9920	$y = 36094x + 99.7$	1.000	18
Cadmium	0–1000	$y = 257x - 8.7$	0.999	0.05
Arsenic	0–100	$y = 210x + 835.5$	0.999	0.11
Lead	0–1000	$y = 543x + 2799.8$	0.997	0.03
Mercury	0–100	$y = 6265x - 540.7$	0.996	0.17

prepared in the ranges of concentrations reported in Table 2. This reports also the coefficients of determination and DLs calculated as $3\sigma/S$ (where σ is the relative standard deviation of noise and S is the sensibility of the measure estimated as the slope of a calibration curve constructed for the isotope considered).

Recovery tests have been carried out by fortification of the samples with the elements determined in such amounts to realise concentrations of 1 mg/kg and 5 mg/kg, corresponding respectively to the acceptable maximum level established from the EFSA (2004) for these metals and to five times as much. Table 3 shows results (expressed as recovery percentage of each metal) obtained by four replicate analysis.

3. Results and discussion

3.1. Method optimisation

The HPLC method used for the analysis of creatine and its impurities was a modification of the method developed by Degussa AG (Manfred Wildenauer, personal communication; Pischel & Gastner, 2007) for internal quality control of creatine monohydrate supplements. First, it was verified if the Degussa method, validated for the detection of creatine, creatinine, dicyandiamide and dihydro-1,3,5-triazine (to test products obtained using sodium or potassium sarcosinate and cyanamide as starting material), was applicable also for the determination of thiourea, a contaminant that can be present using the alternative route from sarcosine and S-methylisothiourea (An et al., 2001). Fig. 1 shows the HPLC traces obtained applying the Degussa method to a standard of thiourea and to a standard mixture of creatine, creatinine, dicyandiamide and dihydrotriazine (UV detection at 225 nm). Creatine and all researched contaminants were well separated and thiourea was the first peak eluted.

The absorbance wavelengths were then optimised for each researched analyte. Creatine has a maximum absorbance wavelength at 210 nm and its absorbance decreases rapidly at higher wavelengths. Dicyandiamide and thiourea show maximum absorbance at 215 nm, creatinine at 215–220 nm and dihydro-1,3,5-triazine at 240 nm. Wavelength changes were programmed during the chromatographic run in order to maximise the response of each contaminant and to lower the response of creatine (230 nm) thus allowing for the injection of more concentrated samples (300 mg/100 ml), without saturating the detector response. This allowed us to slightly improve the detection limits for all the contaminants, particularly for dihydro-1,3,5-triazine, satisfying the purity requirement suggested by EFSA in 2004 (dihydrotrazine < 4.5 mg/kg). By increasing the flow rate from 1.0 to 1.3 ml min^{-1} it was possible to speed up peak elution without loosing in separation efficiency. With the optimised conditions all the analytes eluted in about 8 min.

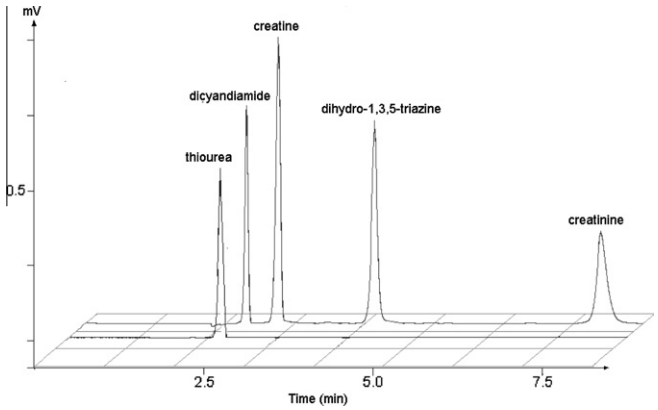

Fig. 1. HPLC traces (UV 225 nm) obtained for thiourea, dicyandiamide, creatine, dihydro-1,3,5-triazine and creatinine applying the method described by Pischel and Gastner (2007).

To avoid interference problems for samples of CrC in formulation with other ingredients (CrC1 and CrC2), it was necessary to opportunely adjust the pH of the mobile phase (3.8 instead of 4.0). CrC1 and CrC2 mainly comprise a large number of ingredients in their formulation: carbohydrates (dextrose, fructose, maltodextrines), artificial sweeteners (aspartame, acesulfame K), amino acids or their derivate (for example L-glutamine, taurine), acidity correctors (citric acid, sodium bicarbonate) and flowerings. By using the mobile phase adjusted at pH 4.0 the creatinine peak partially coeluted with an unknown peak. The list of the ingredients present in both the CrC1 and CrC2 samples allowed us to restrict the number of possible interferents and, with the aid of available standards, it was possible to identify the unknown interferent as aspartame. Fig. 2 shows the HPLC traces obtained for the CrC2 sample using a mobile phase adjusted to pH 4.0 (creatinine and aspartame peaks are only partially separated), pH 4.2 (creatinine coelutes with aspartame) and pH 3.8 (creatinine and aspartame are well separated). No interference by other ingredients present in the formulation was observed in the chromatographic traces of the CrC samples.

Table 3
Recovery of heavy metals.

Analyte	Isotope	% Recovery ± SD (spiked with 1 mg/kg)	% Recovery ± SD (spiked with 5 mg/kg)
Cadmium	114	96.3 ± 6.4	104.8 ± 6.1
Arsenic	75	87.6 ± 5.1	89.8 ± 3.0
Lead	202	56.4 ± 7.3	60.8 ± 4.7
Mercury	208	61.7 ± 4.5	66.2 ± 3.7

3.2. Creatine content

To assess product purity, creatine percent expressed as creatine anhydrous (w/w) was calculated and reported in Table 4 for all the products analysed. The CrM samples showed a creatine content ranging from a minimum of 78.6% to a maximum of 90.9%, with

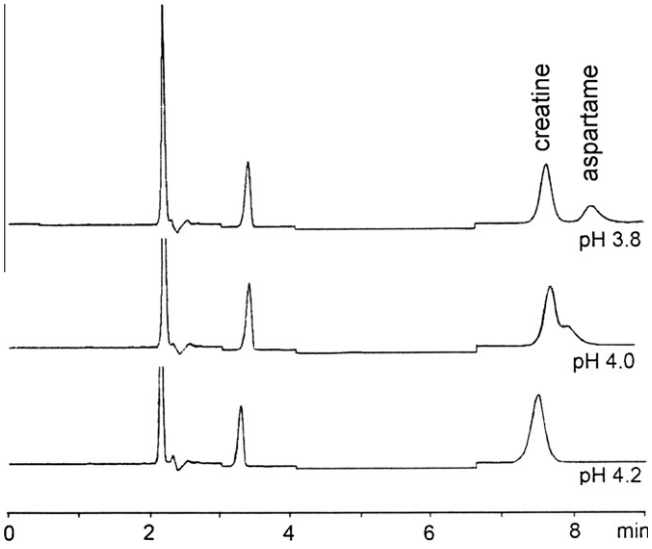

Fig. 2. Effect of the pH of the mobile phase on the separation of creatinine and aspartame.

an average of 87.1%. Considering that CrM has an average water content around 12.1% (Weiss & Krommer, 1998), its theoretical creatine content is 87.9%. Higher creatine content, as those reported in Table 4, could be explained by major dehydration during creatine production.

Most of the CrM products analysed were labelled as pure creatine monohydrate (100%). The percent of CrM in products based only on CrM (without other ingredients) varied from 88% to 102%. Considering an analytical tolerance of ±15%, established by the Italian legislation (Ministry of Health, Circular No. 7 of 30 October 2002), all the products had a CrM content in agreement with that declared on the label.

The two CrM samples in formulation with other ingredients (CrM20 and CrM22) contained different percent of creatine anhydrous (67.0% for CrM20 and 23.4% for CrM22). Sample CrM20 was labelled as 76.9% CrM, which corresponds to 67.6% of creatine anhydrous (there was therefore a good agreement with the value reported on the label). No indication about the percent of CrM present in the product was reported for sample CrM22.

Compared to the CrM products, the CrA samples had lower creatine content, between 72.7% and 85.9%, with an average of 78.3%. Kre-Alkalyn® is a CrM formulation with maltodextrin, sodium carbonate, magnesium stearate, magnesium glycerol phosphate, flavouring and sweeteners, present in variable percentages, which are not exactly specified in the patent; the patent only gives a range in which they should fall (Golini, 2002). This could explain the high variability of the creatine percent found in these products.

Considering that CrC samples were tri-creatine citrate (which is 65.0% creatine and 35.0% citrate), all CrC samples had a creatine content that agreed well with those reported on the label (29.4% of creatine anhydrous which corresponds to 45.2% of CrC for CrC1 and 19.3% of Cr anhydrous which corresponds to 29.7% of CrC for CrC2 and 66.0% of creatine anhydrous which corresponds to about 100% CrC for CrC3).

Table 4
Percent of creatine anhydrous (w/w) and concentration (mg/kg of product) of organic contaminants and heavy metals in the samples analysed.

Samples	Cr (% w/w)	Thi (mg/kg)	Dcd (mg/kg)	Dht (mg/kg)	Crn (mg/kg)	As (mg/kg)	Cd (mg/kg)	Hg (mg/kg)	Pb (mg/kg)
Creatine monohydrate (CrM)									
CrM1	89.9	<DL	11.1	<DL	77.3	<DL	<DL	<DL	<DL
CrM2	89.5	<DL	14.8	<DL	37.8	<DL	<DL	0.16	<DL
CrM3	78.6	<DL	20.4	<DL	97.7	<DL	<DL	<DL	<DL
CrM4	87.5	<DL	13.6	<DL	65.8	<DL	<DL	<DL	<DL
CrM5	86.8	<DL	14.9	<DL	34.7	<DL	<DL	<DL	<DL
CrM6	80.9	<DL	3.9	<DL	163.0	<DL	<DL	<DL	<DL
CrM7	85.8	<DL	14.79	<DL	39.6	<DL	<DL	<DL	<DL
CrM8	84.6	<DL	19.4	<DL	54.6	<DL	<DL	<DL	<DL
CrM9	87.1	<DL	21.0	4.64	51.9	<DL	<DL	0.10	<DL
CrM10	84.9	<DL	20.6	<DL	76.3	<DL	<DL	0.12	<DL
CrM11	90.9	<DL	19.6	<DL	57.8	<DL	<DL	0.12	<DL
CrM12	87.3	<DL	16.6	<DL	62.9	<DL	<DL	0.16	<DL
CrM13	89.9	<DL	42.7	<DL	62.8	<DL	<DL	0.09	<DL
CrM14	90.4	<DL	29.3	<DL	63.5	<DL	<DL	0.10	<DL
CrM15	87.7	<DL	<DL	<DL	259.1	<DL	<DL	0.19	<DL
CrM16	89.0	<DL	3.9	8.0	3499.8	<DL	<DL	0.11	<DL
CrM17	88.6	<DL	12.4	<DL	54.6	<DL	<DL	0.11	<DL
CrM18	88.7	<DL	17.9	<DL	31.7	<DL	<DL	0.11	<DL
CrM19	88.4	<DL	13.3	<DL	45.9	<DL	<DL	0.10	<DL
CrM20	67.0	<DL	17.7	<DL	371.6	<DL	<DL	0.10	<DL
CrM21	90.0	<DL	20.6	<DL	45.4	<DL	<DL	0.09	<DL
CrM22	23.4	<DL	4.6	<DL	1211.6	<DL	<DL	0.10	<DL
CrM23	84.7	<DL	5.4	<DL	180.9	<DL	<DL	0.12	<DL
Kre-Alkalyn® (CrA)									
CrA1	75.6	<DL	6.8	<DL	881.6	<DL	<DL	0.10	<DL
CrA2	85.9	<DL	67.7	4.5	179.7	<DL	<DL	0.11	<DL
CrA3	72.8	<DL	82.2	5.6	92.3	<DL	<DL	0.09	<DL
CrA4	82.6	<DL	66.4	6.4	395.4	<DL	<DL	0.09	<DL
CrA5	80.3	<DL	63.2	<DL	117.9	<DL	<DL	<DL	<DL
CrA6	72.7	<DL	49.2	<DL	258.5	<DL	<DL	0.05	<DL
Creatine citrate (CrC)									
CrC1	29.4	<DL	3.1	<DL	274.5	<DL	<DL	0.07	<DL
CrC2	19.3	<DL	6.1	<DL	155.9	<DL	<DL	0.09	<DL
CrC3	66.0	<DL	2.9	<DL	512.6	<DL	<DL	0.16	<DL

Cr = creatine, Thi = thiourea, Dcd = dicyandiamide, Dht = dihydro-1,3,5-triazine, Crn = creatinine, DL = detection limit.

3.3. Concentration of organic contaminants

Concentrations (mg/kg) of organic contaminants are reported in Table 4 together with the percent of creatine anhydrous. All data are the mean of two replicate samples. The contamination level observed for CrM products were lower than those reported by Brink (1998, 1999) and comparable to those quoted by Harris (2001).

Only five samples (CrM9, CrM16, CrA2, CrA3, CrA4) had dihydro-1,3,5-triazine concentrations exceeding the detection limit of 4.5 mg/kg, with maximum concentrations (8.0 mg/kg) well below the contamination levels reported by Brink (1998, 1999) (up to 410 mg/kg). Regarding dicyandiamide, the CrC samples had on average the lower contamination levels (between 2.9 and 6.1 mg/kg), while the CrM samples had concentrations varying from values under the detection limit up to 42.7 mg/kg, with an average of 15.6 mg/kg. The major contamination level was observed in the CrA samples which, except for sample CrA1 (6.8 mg/kg), had contamination levels between 49.2 and 82.2 mg/kg. It seems that the more basic is the pH of the product (dissolved in water) the higher is the dicyandiamide concentration. As a matter of fact, cyanamide dimerises in the alkaline range to give dicyandiamide, the maximum reaction rate being at about a pH of 9.6 (Weiss & Krommer, 1998).

Creatinine was the major organic contaminant of creatine supplements. This is probably due to the easiness at which it is formed from creatine in non-optimised production or conservation conditions (in water under acidic conditions or at high temperatures). CrM supplements showed a high variability in the creatinine contents with levels between 31.7 and 3499.8 mg/kg. Six out of the 23 tested samples (about 25% of total CrM samples) contained creatinine levels above 100 mg/kg (maximum acceptable value). In particular two samples had exceptionally high creatinine levels (CrM16 with 3500 mg/kg and CrM22 with 1212 mg/kg). Except for sample CrA4, CrA and CrC samples had always creatinine content exceeding 100 mg/kg, between 92.3 and 881.6 mg/kg for CrA, and between 155.9 and 512.6 mg/kg for CrC.

It is interesting to note that the technical data sheet of tri-creatine citrate indicates a maximum creatinine content of 500 mg/kg, which is more in accordance with the results found in the samples analysed. Probably, due to the higher instability of creatine at acidic pH, it is difficult to guarantee for this form of creatine a creatinine content lower than 100 mg/kg.

Another remark can be made about this contaminant, for products based on CrA. In fact, this relatively new creatine form was patented with the objective of reducing the formation of creatinine in water solution (Golini, 2002). Nevertheless, as observed from these results, the CrA samples had on average a higher creatinine content than the CrM samples.

Since none of the products analysed revealed the presence of thiourea, while all except one (CrM15) contained detectable amounts of dicyandiamide, it can be concluded that none of the samples were synthetised from sarcosine and S-methylisothiourea (An et al., 2001).

In 1999, Brink reported a higher contamination levels in the CrM supplements from USA producers compared to European producers. From the indications reported on the label it was verified that 10 out of the 23 samples of CrM were produced in Europe, four in the USA, while for the remaining nine samples it was not possible to establish the provenience. Even though the two groups of provenience were not homogeneous for a number of samples, they showed comparable median values for both creatinine and dicyandiamide (20 and 16 mg/kg for creatinine in CrM supplements from USA and EU producers, respectively; 65 and 60 mg/kg for dicyandiamide in CrM supplements from USA and EU producers, respectively).

As reported in the EFSA opinion (2004), the shelf life of CrM is minimum 36 months from the date of manufacture (stored in an unopened container at room temperature). Elevated creatinine levels can also be found in creatine products that have surpassed their expiration date (Franco-Obregón, 2007). No correlation was found between the residual shelf life (time to the expiration date) and the creatinine content of different products analysed. As an example, samples CrM5 and CrM7 with a residual shelf life of 1 and 2 months, respectively, had a creatinine content lower than 40 mg/kg, while sample CrM15 with a residual shelf life of 32 months had a creatine content of about 260 mg/kg. This seems to suggest that the powder (stored in unopened containers) is stable for a long time and that probably most of the creatinine found is already present in the product before packing.

3.4. Metal concentration

The ICP-MS analysis of the searched heavy metals is reported in Table 4. As observed, considering the detection limits of the instrumental technique, all of the analysed samples were lacking As, Cd and Pb. Only Hg was found in detectable amounts when the instrument was coupled to a hydride generator. Eight products were lower than the DL (0.03 mg/kg of product) obtained for such a metal, 12 were in the interval between 0.03 and 0.10 mg/kg and 14 were over 0.10 mg/kg. The maximum of 0.19 mg/kg was detected for the CrM15 sample. Comparing the found pollution levels with the limit of 1 mg/kg defined for Hg (EFSA, 2004) it can be observed that, also for this metal, all the samples had a lower amount of Hg.

4. Conclusions

A sensible and reliable HPLC method has been optimised for the simultaneous determination of creatine and four possible organic contaminants (creatinine, dicyandiamide, dihydro-1,3,5-triazine and thiourea) in different types of creatine supplements (CrM, CrC and CrA), commercialised both in their pure form or in formulation with other ingredients. All the CrM and CrC products had a creatine content in agreement with that declared on the label.

Regarding the CrA samples, it was not possible to verify the product purity as other ingredients present in variable percentages were not exactly specified in the patent or in the label. With respect to the situation reported in previous works lower but still high concentration of organic contaminants have been reported for the CrM supplements. The CrA samples were in general the most contaminated.

In general, 50% of the analysed products exceeded the maximum level recommended by EFSA (2004) for at least one contaminant. In particular, only 5 (three samples of CrA and two samples of CrM) out of the 33 samples analysed had dihydro-1,3,5-triazine concentrations exceeding the detection limit of 4.5 mg/kg (maximum 8.0 mg/kg) and none of the samples contained detectable amount of thiourea (as all the products analysed were obtained from the synthetic route which starts from sodium or potassium sarcosinate and cyanamide). Creatinine was the most widespread organic contaminant with amounts often exceeding the limit recommended by EFSA (100 mg/kg). Among the different product type, CrC and CrA had on average the largest contamination levels. The CrA products were also the most contaminated with dicyandiamide (levels over 50 mg/kg for five out of the six samples analysed), while CrC had the lowest contamination level. Possible contamination with heavy metals seems to be not of particular concerns.

Considering the results of this study and the lack of official control for the presence of creatinine in creatine supplements it is

advisable that consumers give their preference to products obtained by producers that ensure the highest quality control and certify the maximum amount of contaminants present in their products.

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