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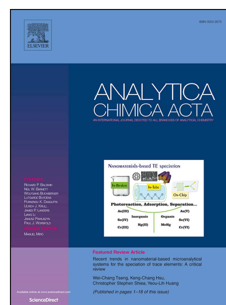

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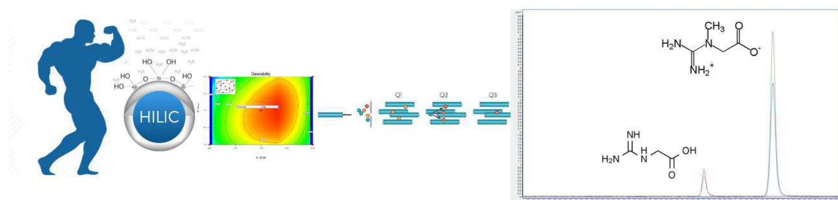

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Hydrophilic interaction chromatography coupled to tandem mass spectrometry as a method for simultaneous determination of guanidinoacetate and creatine

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26
Abstract

27 The biosynthesis of creatine (Cr) is closely related to the bioavailability of guanidinoacetate (GAA). The
28 lack of one or the other may compromise their role in the energy transport and cell signaling. A reliable
29 estimate of their levels in biological samples is imperative since they are important markers of many
30 metabolic disorders. Therefore, a new LC-MS/MS method for simultaneous determination and
31 quantification of GAA and Cr by multiple reaction monitoring (MRM) mode was developed based on the
32 hydrophilic interaction chromatography (HILIC) and response surface methodology (RSM) for the
33 optimization of chromatographic parameters. The optimized parameters ensured good separation of these
34 similar, very polar molecules (chromatographic resolution > 1.5) without prior derivatization step in a
35 short analysis run (6 min). The developed method was validated to ensure accurate (R, 75.1–101.6%),
36 precise (RSD < 20%) and low quantification (LOQ of 0.025 μg mL^{-1} for GAA and 0.006 μg mL^{-1} for Cr)
37 of the tested analytes and the use of matrix-matched calibration eliminated variable effects of complex
38 matrices such as human plasma and urine. Therefore, this method can be implemented in medical
39 laboratories as a tool for the diagnostics of creatine deficiencies and monitoring of guanidinoacetate and
40 creatine supplementation regimes in biological samples.

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44 *Keywords: Creatine; Guanidinoacetate; LC-MS/MS; HILIC; RSM*

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51 # 1. Introduction

52

53 Creatine (Cr) is a natural guanidine-derived compound responsible for the maintenance of cellular
54 bioenergetics [1]. In humans, half of the needed Cr is produced endogenously (mainly in the liver,
55 kidneys and pancreas) and a half can be supplied through diet (mainly from meat or fish) or through Cr
56 supplementation with reduced endogenous production [2, 3]. Creatine biosynthesis starts in the kidneys,
57 where arginineglycine amidinotransferase (AGAT) catalyzes the formation of ornithine and
58 guanidinoacetate from glycine and arginine. Thereafter, guanidinoacetate (GAA) is transferred to the liver
59 where it receives a methyl group from S-adenosylmethionine to form a creatine in the reaction catalyzed
60 by guanidinoacetate methyltransferase (GAMT) [2]. Due to the fact that GAA has the central role in Cr
61 synthesis, acting as a direct precursor that requires only a methyl-group transfer, supplementation with
62 GAA could directly restore the Cr load in tissues [4]. Levels of both Cr and GAA in physiological fluids
63 can be reliable diagnostic markers for primary creatine deficiency syndromes (AGAT, GAMT) and
64 secondary creatine deficiencies that may occur in other metabolic diseases related to creatine biosynthesis
65 [5]. Furthermore, some types of muscle disorders, such as primary myopathy, myositis, muscle atrophy
66 and hyperthyroidism can be distinguished by elevated serum levels of creatine [6, 7]. Lack of creatine in
67 the central nervous system can cause severe neurological impairments such as developmental delay,
68 hypotonia, involuntary movements, delay or lack of speech acquisition, mental retardation of variable
69 severity, autistic behavior and epilepsy [8, 9]. Replacement therapy with Cr and GAA seems to respond
70 positively to overall creatine pool storage in the body and biosynthesis deficiencies [10-12].

71 Since its introduction as a dietary supplement on the global sport supplements market, creatine
72 has become very popular among athletes due to numerous studies proving its positive benefits related to
73 enhancement of exercise performance, reducing fatigue, accelerating energy recovery and muscle growth,
74 increasing muscle strength and promoting muscle size without affecting body fat [3, 13-16]. Compared
75 to Cr, the use of GAA as a supplement has been only approved in animal nutrition due to its

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76 association to hyperhomocysteinemic effects as reported while studying its efficiency and safety
77 after exogenous GAA administration in humans [17]. However, there might be a possibility for
78 GAA supplementation in humans, since most recent studies have shown positive correlations
79 between GAA supplementation and an increase of brain creatine levels with no signs of
80 neurotoxicity [4], creatine availability, utilization in tissues with high-energy requirements [17]
81 and positive achievements in a treatment of heart, kidney and neuromuscular diseases [12].

82 The necessity of diagnosis of low concentration of Cr and GAA results in the development of a
83 wide range of analytical methods for the determination of Cr and GAA in biological samples. Several
84 analytical methods have been utilized for the determination of guanidinoacetate and/or creatine in
85 biological samples and sports supplements products, including gas chromatography-mass spectrometry
86 (GC-MS) [14, 18, 19], liquid chromatography with MS (LC-MS) [7], high-performance LC (HPLC) with
87 fluorescence [20] and UV detection [3, 16] and LC tandem MS (LC-MS/MS) [21-26]. A comparative
88 study done by Arias *et al*. [27] showed a good agreement among chromatographic methods used for
89 determination of GAA in urine. Considering the high polarity and non-volatility of the analytes, as well as
90 the background interference of the biological matrix, all the mentioned methods require sample
91 pretreatment, such as solid-phase extraction that makes sample preparation time-consuming, labor-
92 intensive and more expensive [28, 29] and/or an addition of ion-pairing reagents [5, 8, 13, 30] or an
93 addition of phosphate buffer to mobile phase [31, 32], which could result in contamination of ion source
94 and requires frequent cleaning. The method developed by Carling *et al.* [2] offers the possibility to
95 quantify GAA, creatinine (CR) and Cr by underivatized LC tandem MS spectrometry.

96 Reverse phase liquid chromatography (RP-LC) is the most used separation technique with a
97 major limitation considering low retention of very highly polar molecules such as Cr and GAA. However,
98 the retention of polar analytes often requires a highly aqueous mobile phase sometimes leading to
99 problems such as phase collapse or decreased sensitivity in mass spectroscopic detection due to poor
100 mobile phase desolvation and ion suppression. Although normal-phase liquid chromatography (NP-LC) is

101 often applied to separate polar compounds, an important limitation is a difficulty of dissolving
102 hydrophilic compounds in non-aqueous mobile phases. For these reasons, respecting the environmental
103 aspects, the application of hydrophilic interaction chromatography (HILIC) is an alternative for the
104 separation of Cr and GAA in biological samples. The mechanism of HILIC involves various
105 combinations of hydrophilic interaction, ion exchange, and reversed-phase retention by the siloxane on
106 the silica surface. Furthermore, HILIC provides longer retention of polar compounds with their elution in
107 order to increase hydrophobicity, the use of volatile buffers to avoid desalting step, there is no need of
108 compound derivatization, the nature of the used mobile phases is comparable to reverse-phase and sample
109 processing and compatibility with further mass spectrometry analysis easier [33].

110 In the development of chromatographic methods response surface methodology (RSM) is a
111 widely used approach for the optimization of chromatographic parameters [34]. The main advantage of
112 RSM is a minimal number of acquired experiments necessary for obtaining system information. Quadratic
113 polynomial models with Box-Behnken designs (BBDs), a class of rotatable second-order designs, have
114 been considered as the most appropriate RSM solution for building quadratic response surface to predict
115 the optimized chromatographic parameters. Derringer's desirability presents a multicriteria decision
116 making approach for the simultaneous optimization of several chromatographic parameters, offering
117 flexibility to user in the definition of desirability functions [35-37] and it was employed in the RSM
118 method optimization process for determination of Cr and GAA. In comparison to findings of Carling *et*
119 *al.* [2], it was difficult to get the reproducible results in our laboratory using reverse phase
120 chromatographic column, probably due to high polarity of the investigated analytes and complexity of the
121 sample matrices.

122 A new approach in LC-MS/MS determination of Cr and GAA without prior derivatization step
123 was applied based on hydrophilic interaction chromatography utilizing response surface methodology in
124 development of accurate, rapid and sensitive analytical method.

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126 # 2. Experimental procedure

127

128 ## 2.1. Chemicals and reagents

129 Standard of creatine monohydrate (certified purity as mass fraction ω > 99%) and formic acid (ω
130 = 98%) were purchased from Sigma-Aldrich (Steinheim, Germany), while standard of guanidinoacetate
131 (ω > 99%) and acetonitrile (ACN) of HPLC grade purity were purchased from Merck (Darmstadt,
132 Germany). HPLC grade purity methanol (MeOH) and ammonium acetate were purchased from J.T. Baker
133 (Phillipsburg, USA). Purified water by a Simplicity UV system from Millipore (Bedford, USA) was used.
134 A bicomponent stock solution of GAA and Cr standards (100.0 µg L^{-1}) was prepared in water and stored
135 frozen at -20 °C, staying stable over a period of at least three months. The obtained stock solution was
136 dissolved in water to obtain the final concentrations in the range from the limit of quantification (LOQ) to
137 100 µg L^{-1} for both analytes. These solutions were used for solvent calibration (SC), matrix-matched
138 calibration (MMC) and spiking plasma and urine samples. All working solutions were hermetically sealed
139 and stored in refrigerator (at 4 °C) protected from light assuring stable conditions for at least one month.

140

141 ## 2.2. Sample preparation and metabolites extraction

142 Plasma was obtained from blood samples collected in EDTA K2 tubes and centrifuged for 5 min
143 at 3000 rpm (1260 rcf). After plasma collection, samples were stored frozen at -20 °C.

144 Plasma sample (250 µL) was mixed by a vortex for 30 s with 2 mL of MeOH and left in a
145 refrigerator (at 4 °C) for 20 min, after which the mixture was centrifuged for 15 min at 4000 rpm
146 (2240 rcf). Then, the supernatant was transferred to a clean vial and dried at 40 °C under nitrogen steam
147 and finally reconstituted with 1 mL of chromatographic mobile phase prior to LC-MS/MS analysis.

148 Preparation of urine samples was performed following the procedure: 350 µL of urine sample
149 was mixed by a vortex for 30 s with 1050 µL of MeOH and then centrifuged for 15 min at 4000 rpm. The
150 supernatant was transferred to a clean vial and dried at 40 °C under nitrogen steam and finally

151 reconstituted with the addition of 1 mL of chromatographic mobile phase and mixing for 60 seconds by a
152 vortex. Keeping samples in a dry form prior to reconstitution step prevented any possible conversion of
153 analytes [38].

154

155 2.3. The LC-MS/MS system and operating conditions

156 Separation and quantification of GAA and Cr were achieved with a LC-MS/MS system
157 consisting of Agilent 1200 series HPLC (Agilent Technologies Inc., USA) and a Triple Quad Agilent
158 6410 mass spectrometer (Agilent Technologies Inc., USA). The HPLC system utilized a solvent
159 degassing unit, a quaternary pump, an autosampler and a thermostatted column compartment for the
160 separation of target analytes. Separation was achieved by using a Kinetex HILIC 100A (100 mm × 2.1
161 mm i.d., 2.6 μm, Phenomenex, USA) column with a column temperature of 30 °C. The mobile phase
162 consisted of two eluents, ACN (A) with 0.1% HCOOH and MeOH/H_2O (1:1, v/v) with 100 mM
163 ammonium acetate (pH = 3.7 adjusted by the addition of 0.1% HCOOH) (B), delivered at a flow rate of
164 0.5 mL min^{-1} with isocratic elution (solvent A: solvent B 80:20, v/v) and the run time of 6 min and post-
165 time of 2 min to ensure the equilibration of the system. The injected volume of samples and standards was
166 10 μL.

167 The mass spectrometer was operated with a multimode interface in positive ion mode. Mass
168 Hunter workstations software, version B.03.01 (Agilent Technologies Inc., USA), was used for the
169 control of equipment, data acquisition and analysis. The instrument was operated with the heater gas
170 temperature of 325 °C and vaporization temperature of 200 °C. Nitrogen was used as a nebulizer gas at 50
171 psi, and a flow rate of 5.0 L min^{-1}, capillary voltage of 2500 V and charging voltage of 2000 V were set.
172 Standard solutions (1.0 μg mL^{-1}) of GAA and Cr were injected directly into the mass spectrometer to
173 obtain their transition, precursor, and relevant product ions based on the multiple reaction monitoring
174 mode (MRM). To provide the best response for quantification, optimization of parameters such as
175 fragment, voltage, dwell time, and collision energy was conducted. Each analyte was characterized by its
176 retention time and two precursor-product ion transitions. The most intense product ion was used for

177 quantification, whereas the second most intense product ion was used for the identification. The MRM
178 transitions used a dwell time of 20 milliseconds.
179

180 2.4. Optimization of chromatographic parameters with response surface methodology

181 Straightforward design of a multivariable experimental procedure, which includes experimental
182 design, ANOVA data analysis, and desirability function calculations, was enabled with the use of Design-
183 Expert 7.0.0. (Stat-Ease, Minneapolis, USA) software. Preliminary experiments were conducted prior to
184 selection of factors (eluents' composition, column temperature and flow rate) for the optimization process.
185 Modified method developed by Derringer and Suich [39] was applied in the procedure followed in this
186 work for simultaneous optimization of three responses (resolution of the peaks R_{2-1} creatine-
187 guanidinoacetate and peaks area for creatine and guanidinoacetate individually). Transformation of each
188 predicted response to a dimensionless partial desirability function made it possible to perceive
189 researcher's desires and priorities during the optimization procedure. Geometric mean of the different
190 partial desirability values gave the global desirability function D, with values different from 0 and close to
191 1 implying that all responses are simultaneously in a desirable range and consequently response values
192 are near the target values providing the global optimum. The optimization factors, chosen responses and
193 optimization criteria are presented in Table 1.
194

195 2.5. Method performance

196 Validation of the optimized method for determination of GAA and Cr comprehended a check of
197 all the necessary validation parameters to ensure reliability and quality of the proposed analytical method.
198 The calibration curves in pure solvent as well as in plasma and urine matrices (5.0, 10.0, 25.0, 50.0, 75.0
199 and 100.0 μg L^{-1}) were obtained by plotting the peak areas against the concentrations of the
200 corresponding calibration standards on six levels, expressing the linearity of calibration curves by the
201 square correlation coefficient (r^2). The limit of detection (LOD) and limit of quantification (LOQ) were

202 estimated by injecting decreasing concentrations of matrix-matched standards and measuring the response
203 at a signal-to-noise ratio (S/N) of ≥ 3 and ≥ 10 for the LOD and LOQ, respectively. The matrix effect,
204 which is a phenomenon in mass analysis, has either an ion suppression or enhancement effect. In this
205 study, the matrix effect was assessed by comparing the signal responses in the pure solvent solution and
206 in plasma and urine matrices. To ensure the specificity and selectivity of the method, the possibility of
207 interfering compounds was investigated by analyzing matrix-matched samples prepared using a blank
208 sample (known to contain neither of the analyzed compounds) to ensure that there was no interference at
209 the GAA and Cr retention times. Due to the unavailability and cost effectivity of the isotopically labeled
210 standards, the accuracy of the method was determined with the percentage recovery (R, %) of spiked
211 blank plasma and urine samples prior to analysis using the method of standard addition (at 3
212 concentration levels) calculated using matrix-matched calibration curves. The precision was expressed in
213 terms of method repeatability (the analysis was performed on the same day (n=5) with the same
214 instrument and the same operator) and within-laboratory reproducibility (three different days with the
215 same instrument and by different operators) as relative standard deviation (RSD), targeting precision
216 values below 20%.
217
218 # 3. Results and discussion
219
220 High-performance liquid chromatography (HPLC) has been widely used for determination of the
221 aforementioned compounds in biological samples. Considering the high polarity and non-volatility of the
222 analytes, as well as the background interference of the biological matrix, liquid chromatographic analysis
223 of these energy-related biomolecules presents a challenge. There were two main innovative approaches to
224 answer this challenge presented in this study compared to other published methods: a) the employment of
225 hydrophilic interaction chromatography for the separation of GAA and Cr (chromatographic resolution >
226 1.5) in complex matrices such as plasma and urine samples in short analysis time with isocratic elution

227 regime and b) the employment of response surface methodology for the optimization of the
228 chromatographic parameters of the developed analytical method.
229
230 3.1. Optimization of chromatographic parameters
231 Creatine and guanidinoacetate are very polar compounds and this is quite a challenge in
232 separation analysis. Preliminary experiments were conducted with the purpose of finding the optimal
233 instrumental conditions that would allow good LC separation with high sensitivity and unambiguous
234 MS/MS identification and quantification of investigated molecules at low concentration levels. Mixed
235 standard solution (100.0 µg L^{-1}) was used for this optimization test. Different mobile phase flow rates,
236 eluent composition and column temperatures were tested in order to determine the key factors for the
237 optimization process in regard to achieve the best separation during a short run time. The optimization of
238 the eluent composition during the analysis run and the flow rate, together with the column temperature
239 was done by using response surface methodology with Box-Behnken designs combined with Derringers
240 desirability function [37, 40]. The investigation of the associated probability revealed that for all tested
241 responses quadratic models resulted as the best fit. Response surface plots representing quadratic effects
242 of eluent composition and flow rates at set up column temperatures on target peaks area and peak
243 resolution are shown in Figure 1.
244 The statistical data for the models obtained from ANOVA are presented in Table 1. The
245 correlation coefficients were obtained within acceptable limits of $r^2 \geq 0.90$, which pointed out that the
246 experimental data fitted to the second-order polynomial equations. Values of $p < 0.05$ were obtained for
247 all the models, implying that the models were significant. A measure of the 'signal (response) to noise
248 (deviation) ratio' was expressed as the value of the adequate precision with desirable ratio > 4. In this
249 study adequate precision had a ratio in the range of 6.17 to 20.34, which indicates an adequate signal and,
250 therefore, the model is significant for the given process. Reproducibility of the model measured as RSD
251 can be regarded as reasonably reproducible if the RSD is < 10%, which was achieved for all the models in
252 this study. For all the fitted models satisfied assumptions of normality and constant variance of the

253 residuals were revealed by observing diagnostic plots such as the normal probability plot of residuals and
254 a plot of residuals vs. predicted values. The optimization procedure was carried out based on the
255 conditions and desirable criteria resulting in the optimum conditions with the maximum global
256 desirability value, D = 0.96. The coordinates of D represent the optimum chromatographic parameters
257 with 80% of eluent A with flow rate of 0.5 mL min^{-1} at 30 °C column temperature in 6 min run predicting
258 the satisfactory responses. Figure 2 shows the corresponding response surface plot with optimal values.
259 The utilization of these optimal conditions resulted in better chromatographic resolution between GAA
260 and Cr chromatographic peaks compared to underivatized chromatographic separation found by Carling
261 *et al*. [2].

262 After optimization of the LC conditions it was important to accomplish the optimum transitions
263 and associated acquisition parameters in the MRM modes using the MassHunter optimizer software
264 (Agilent Technologies Inc., USA). For the quantitative mass spectrometric detection, a performance
265 criterion of a minimum three identification points was established. Better sensitivity, as well as better
266 resolution and peak shape were accomplished with the addition of formic acid which in positive
267 ionization substantially promoted the formation of $[M^{+}H]^{+}$ precursor ion. Fragmentor voltage in the range
268 of 80–150 V (in 10 V increments) was tested together with various collision energies (5, 10, 15, 20, 25,
269 and 30 V). The MS/MS obtained optimization parameters (Q1, Q3, FV, and CE) are listed in Table 2 with
270 the lists of precursor and product ions monitored. Due to the complexity of the investigated matrices
271 which usually contain high amounts of matrix co-extractives in order to prevent the electrospray source
272 from contamination, a divert valve was used between the analytical column and mass spectrometer which
273 allowed the flow to pass through the mass spectrometer only during analyte elution and made it possible
274 to analyze a high number of samples without having to clean the source of mass spectrometer.

275

276 3.2. Matrix effects

277 Signal suppression or enhancement of the studied analytes troubleshoots the LC-MS/MS
278 detection due to matrix effects. The use of MMC standards compensates the matrix effect, which is

279 expressed as the signal from the GAA and Cr in the matrix (plasma and urine) compared to the signals in
280 the solvent and calculated as signal suppression/enhancement (SSE), i.e. slope ratio for MMC and SC.
281 Both analytes have shown signal suppression caused by the matrix effects, especially GAA (SSE, 73% for
282 plasma and 82% for urine matrices) exhibited higher level of suppression compared to Cr (SSE, 83% for
283 plasma and 88% for urine matrices) caused by co-extracted polar compounds found in matrices.
284 Furthermore, the matrix effect caused by the compounds found in plasma samples was higher than the
285 effect caused by the compounds found in urine samples. This can be explained by more complex
286 composition of plasma than urine. Regarding indicated matrix effects for the precise quantification of
287 GAA and Cr in biological samples, the use of matrix-matched standards is required. Compared to the
288 study by Wang *et al.* [21] who did not find any obvious matrix effects, and some studies conducted with
289 no special focus on matrix effects [2, 5, 8, 29], the observed SSE found in this study will provide the
290 additional information about the effects that plasma and urine matrixes have on the LC-MS/MS analysis.
291 Due to the possible influence that GAA and Cr may have, as metabolites of CR, on its concentration
292 normalization of obtained values to creatinine was not applied.

293

294 3.3. Method validation

295 After the implementation of the optimized chromatographic parameters the validation of the
296 proposed method was carried out (Table 3). Under these parameters retention times (t_r) for GAA and Cr
297 were constant with the RSD not exceeding 0.1%.

298 The matrix-matched calibration curves were linear over the range LOQ–100.0 μg L^{-1} for the
299 analyzed compounds in both investigated matrices. Linearity, tested using the least square regression
300 method, gave values of r^2 above 0.99 in all the linear ranges. Specificity was demonstrated by identifying
301 the analytes based on the precursor and product ions as well as the relative retention times (compared to
302 the standards). Ion ratios of the investigated analytes typically matched to each other within 10% in both
303 matrix-matched samples and standards in solvent. Chromatographic peaks with acceptable S/N ratios

304 were obtained at the retention times of GAA and Cr with no interfering peaks obtained at the respective
305 retention times in the blank sample assuring good selectivity of the proposed method.

306 The described method is characterized by high selectivity and linearity over large concentration
307 ranges and with high sensitivity for GAA and Cr molecules, comparable with other published LC-MS
308 methods [2, 5, 7, 8]. The extracted and overlapped MRM ion chromatogram of the blank and spiked
309 plasma sample with GAA and Cr mixture at 100.0 μg L^{-1} concentration level is shown in Figure 3. The
310 chromatogram shows good selectivity and chromatographic characteristics of the proposed method.

311 Accuracy of the method was evaluated by the recoveries of spiked plasma and urine samples and
312 precision was evaluated using relative standard deviations (RSDs). Three levels of mixture standard
313 solutions were added to each sample and then analyzed using the established method and the MMC
314 curves. Each level was performed in six replicates. The results of the accuracy of the proposed method
315 were expressed as the mean recovery (R, %) and are shown in Table 3. These results confirmed that the
316 optimal recovery (70–120% at each spiking level with RSD of ≤ 20%) was obtained for all investigated
317 GAA and Cr concentration levels in both types of matrix. The average recoveries for the three spiked
318 levels were from 75.1% to 101.6%.

319 Precision, expressed as the repeatability and within-laboratory reproducibility gave RSD values
320 for Cr within the range of 1.28–8.40% and 3.45–10.24%, respectively for urine samples and 2.38–9.28%
321 and 5.42–11.84%, respectively for plasma samples. By the same principle, method repeatability and
322 within-laboratory reproducibility for GAA determination was in the range of 2.48–7.44% and 4.42–
323 11.27%, respectively for urine samples and 3.18–10.54% and 7.12–13.64%, respectively for plasma
324 samples. These results indicate a good precision of the developed method.

325 The calculated LODs and LOQs using matrix-matched calibration curves are shown in Table 4.
326 The reached LOQs were at the similar concentration levels as the ones determined by Boenzi *et al.* [5]
327 and Cognat *et al*. [22] and far lower than the ones found in underivatized LC-MS/MS study of Carling *et*
328 *al.* [2] pointing out the increased sensitivity of the developed method and its application in creatine
329 deficiency diagnostics or monitoring of guanidinoacetate and creatine supplementation.

330

331 3.4. Application of LC-MS/MS method

332 The developed and optimized LC-MS/MS method described in this study was successfully
333 utilized in the determination of plasma and urine guanidinoacetate and creatine concentration levels
334 concerning the increases of brain creatine levels in healthy men after supplementation with GAA,
335 published in a research of Ostojić *et al.* [4], providing a direct successful application of this novel method.
336 These results confirm the ability of this method to determine small changes in GAA and Cr levels
337 in tested matrices and therefore it can be used in routine diagnostics of these molecules.

338

339

4. Conclusion

340

341 The implementation of response surface methodology in method development and liquid
342 chromatography optimization process provided results which clearly indicate a good method specificity
343 and selectivity as well as satisfactory chromatographic separation of guanidinoacetate and creatine in
344 complex matrices such as human urine and plasma. The developed LC-MS/MS method was validated to
345 ensure accuracy, precision and low quantification of GAA and Cr and the use of matrix-matched
346 calibration ensured elimination of variable matrix effect. Until now, there was no developed LC–MS/MS
347 method in multiple reaction monitoring (MRM) mode reported on simultaneous determination of GAA
348 and Cr that utilizes the response surface methodology for the optimization of chromatographic method
349 and the hydrophilic interaction chromatography for the separation of these very similar and polar
350 compounds in complex matrices. In addition, this assay offers very fast, reliable and inexpensive method
351 as a tool for the diagnostics of creatine deficiencies and monitoring of guanidinoacetate and creatine
352 supplementation regime in human plasma and urine samples.

353

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503 # Figure captions

504 **Figure 1.** Response surface plots representing quadratic effects of eluent composition and flow rates at
505 set up column temperature on target a) peaks area of GAA, b) peaks area of Cr and c) peak resolution.
506 **Figure 2.** Response surface plot with optimal chromatographic parameter values at column temperature
507 of 30 °C.
508 **Figure 3.** The extracted and overlapped MRM ion chromatogram of the blank and spiked plasma sample
509 with GAA (t_r ~ 3,42 min) and Cr (t_r ~ 4,73 min) mixture at 100.0 µg L^{-1} concentration level.
510

511 # Table captions

512 **Table 1.** Optimization factors, responses, criteria and ANOVA results of the Box-Behnken design.
513 **Table 2.** The m/z of precursor ion (Q1), m/z of monitored product ion (Q3), fragmentor voltage (FV)
514 and collision energy (CE) of investigated analytes.
515 **Table 3.** Retention times, accuracy and precision parameters of the developed method.
516 **Table 4.** Limits of detection and quantification of guanidinoacetate and creatine in biological fluids
517 blood plasma and urine.

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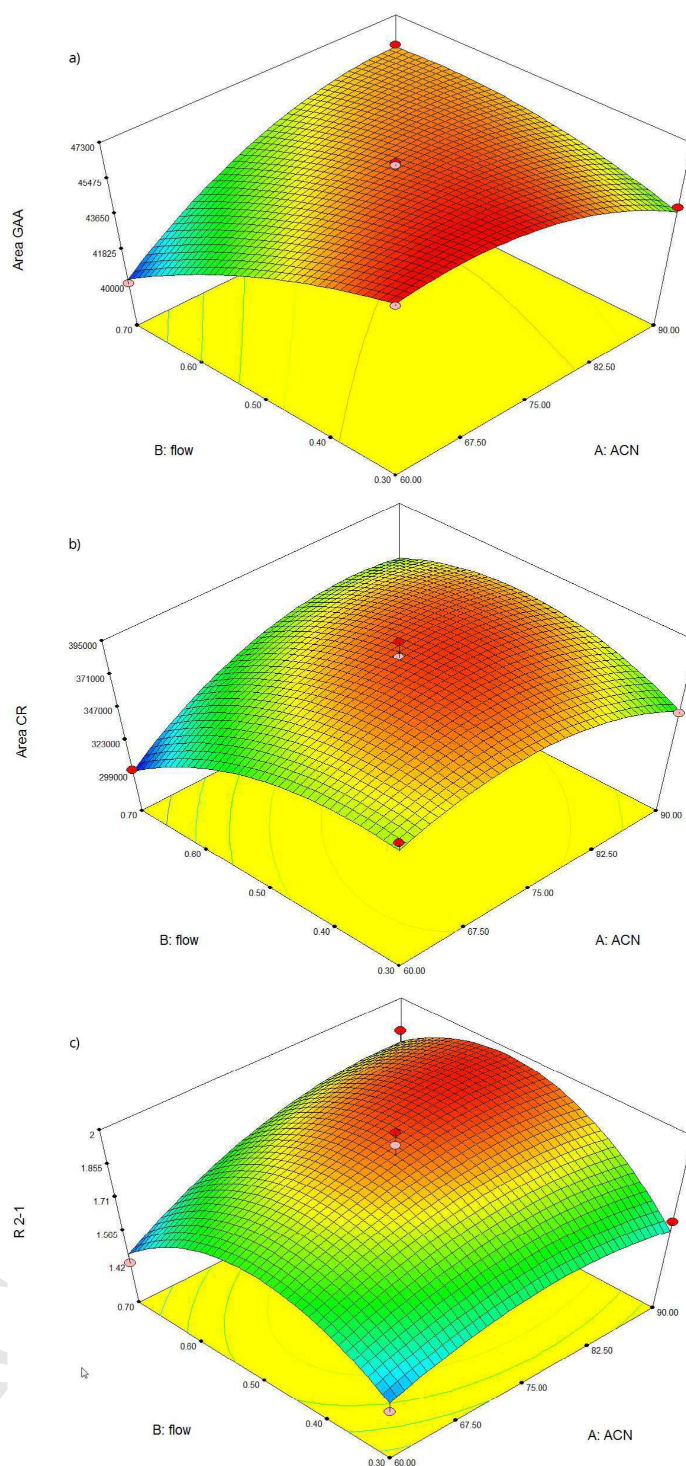

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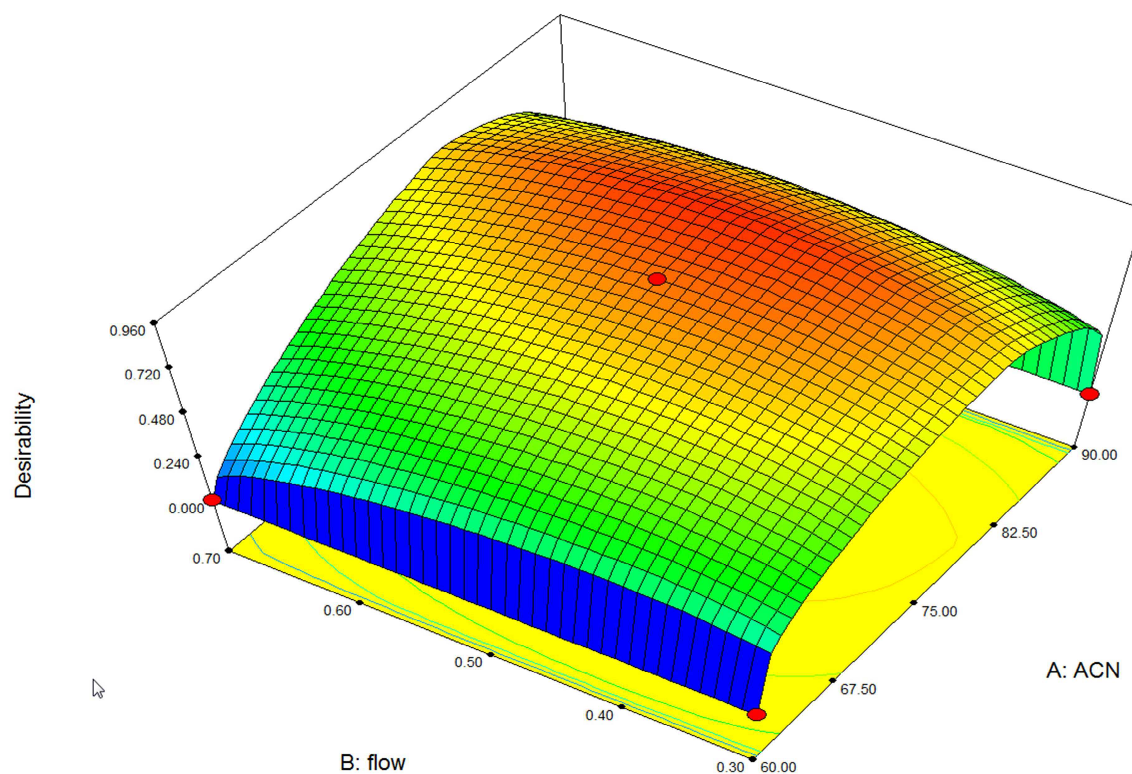

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525 of 30 °C.

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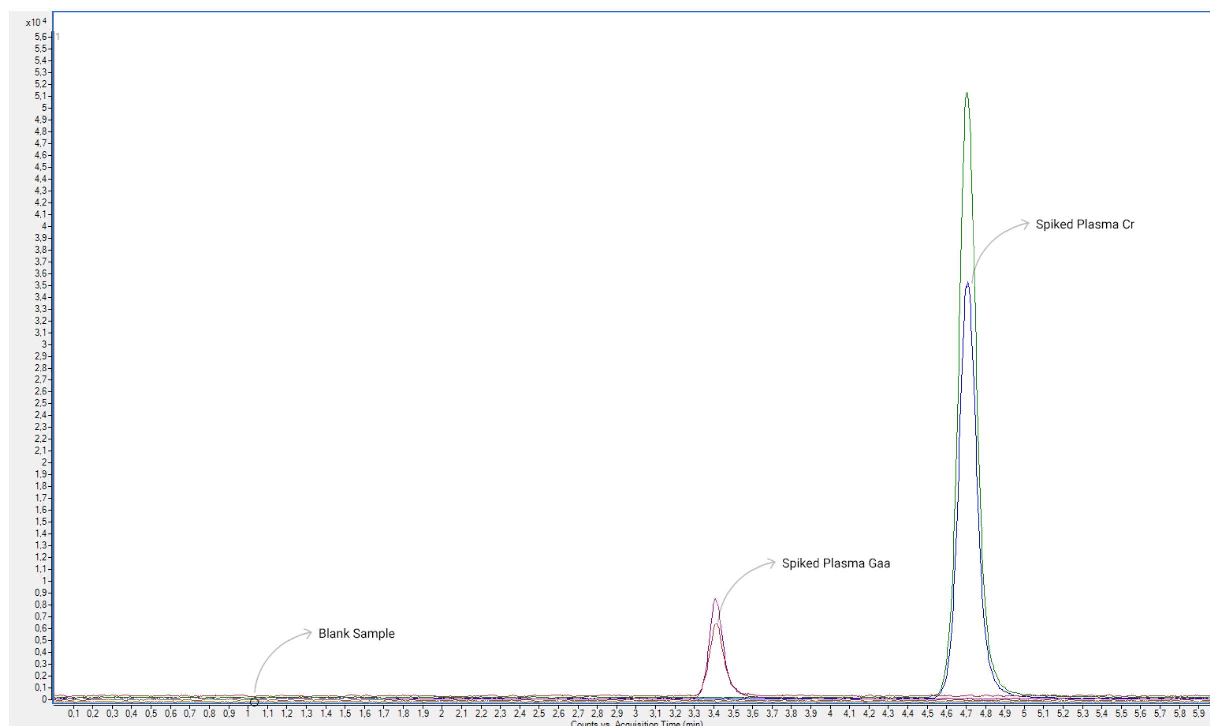

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541 **Table 1.** Optimization factors, responses, criteria and ANOVA results of the Box-Behnken design
542 based on 15 runs.

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Factor	name	low	medium	high	units
A	ACN	60.0	75.0	90.0	%
B	flow rate	0.30	0.50	0.70	mL min^{-1}
C	temperature	20.0	30.0	40.0	°C

Response	model	F-value	p-value	significant model terms	r^2	adequate precision	optimization criteria
Area GAA	quadratic	25.73	0.0011	A, B, AB, A^2, B^2	0.9789	20.341	max
Area Cr	quadratic	15.48	0.0038	A, B, AB, A^2, B^2	0.9654	14.516	max
R_{2-1} (creatine-guanidinoacetate)	quadratic	98.81	0.0424	A, B^2, C^2	0.9031	6.173	≥ 2

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557 **Table 2.** The m/z of precursor ion (Q1), m/z of monitored product ion (Q3), fragmentor voltage (FV)
558 and collision energy (CE) of investigated analytes.
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Analyte	Q1	Q3	FV(V)	CE(V)
GAA	118.1	76.1	90.0	7.0
		100.8	90.0	5.0
Cr	132.1	44.1	100.0	17.0
		90.1	100.0	9.0

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575 **Table 3.** Retention times, accuracy and precision parameters of the developed method.

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Analyte	mean t_r (n=15) (min)	RSD (%) of t_r	Spiking level (μg L^{-1})	Accuracy, mean R (%)		Repeatability (n=5), RSD (%)		Within-laboratory reproducibility (n=3x5), RSD (%)	
				Plasma	Urine	Plasma	Urine	Plasma	Urine
Guadinoacetate	3.41	0.10	100.0	92.4	93.2	3.18	2.48	7.12	4.42
			50.0	95.5	101.6	8.31	5.65	10.29	8.38
			10.0	75.1	87.3	10.57	7.44	13.64	11.27
Creatine	4.78	0.10	100.0	101.1	97.3	4.37	1.28	5.42	9.48
			50.0	84.5	85.5	2.38	7.83	7.20	3.45
			10.0	81.4	79.3	9.28	8.40	11.84	10.24

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591 **Table 4.** Limits of detection and quantification of guanidinoacetate and creatine in biological fluids
592 blood plasma and urine.
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Analyte	LOD (μg mL^{-1})		LOQ (μg mL^{-1})	
	Plasma	Urine	Plasma	Urine
Guanidinoacetate	0.015	0.010	0.050	0.025
Creatine	0.002	0.002	0.010	0.006

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1 **Highlights**

2 ► A LC-MS/MS method for determination of guanidinoacetate and creatine is proposed.

3 ►Separation of analytes was based on Hydrophilic Interaction Chromatography

4 ►Mathematical RSM was used to provide optimal experimental conditions.

5 ► LC-MS/MS provided fast and sensitive quantification method.

6 ► Good validation results make the method applicable for the laboratory practice.

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