APPENDIX 1

Validation rapport Tier 1 screening for GAMT-D using Perkin Elmer Qsight 220

Currently, screening for GAMT-D is not part of any tier 1 newborn screening kit. In this rapport is evaluated whether the hallmark compounds of this disease, creatine and guanidinoacetate (GAA) could be added to the Perkin Elmer Neobase II kit that is currently in use in The Netherlands for tier 1 screening of various inborn errors of metabolism. In addition, an "in-house" method could be design with adequate performance characteristics for tier 1 screening for GAMT-D. The Perkin Elmer Qsight 220 is the mass spectrometer was used for these tests since it is currently in use in the screening laboratories. To evaluate whether another brand of mass spectrometers would be an option, a limited study with an Applied Biosystems API5000 was performed.

1 Optimization

First step in testing new methods is optimizing the parameters of the mass spectrometer to assess whether measurement of desired compounds will be feasible. During the optimization of the GAMT-D method, FIA-MS/MS of 1/8 inch BSP, using the same protocol that is currently used in the Dutch newborn screening turned out to be not feasible due to a combination of severe suppression of the GAA signal by the BSP matrix and the low concentrations of this compound (Figure 1).

FIA-MS/MS was possible for creatine but this is not the primary compound of interest for GAMT-D. Introduction of an UPLC column lowered the suppression to an extent that GAA was measurable (Figure 2).

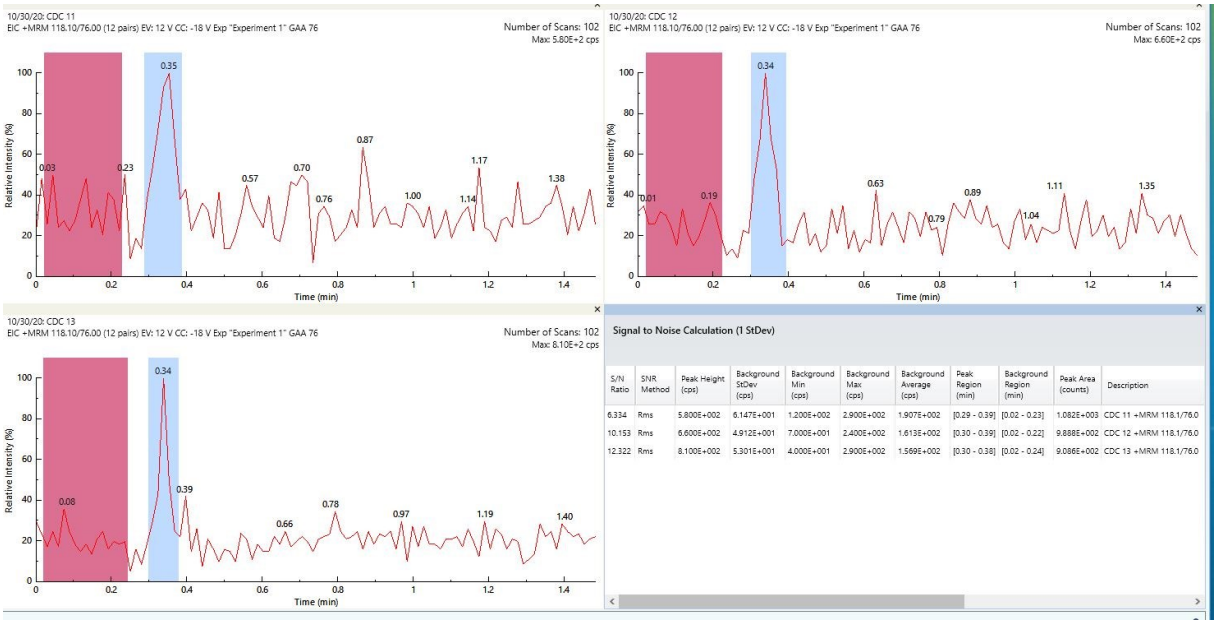


Figure 1 Lowest control BSP for GAA (~ 1 µmol/L) for FIA-MS/MS (S/N ~ 10) measured on a Perkin Elmer Qsight 220.

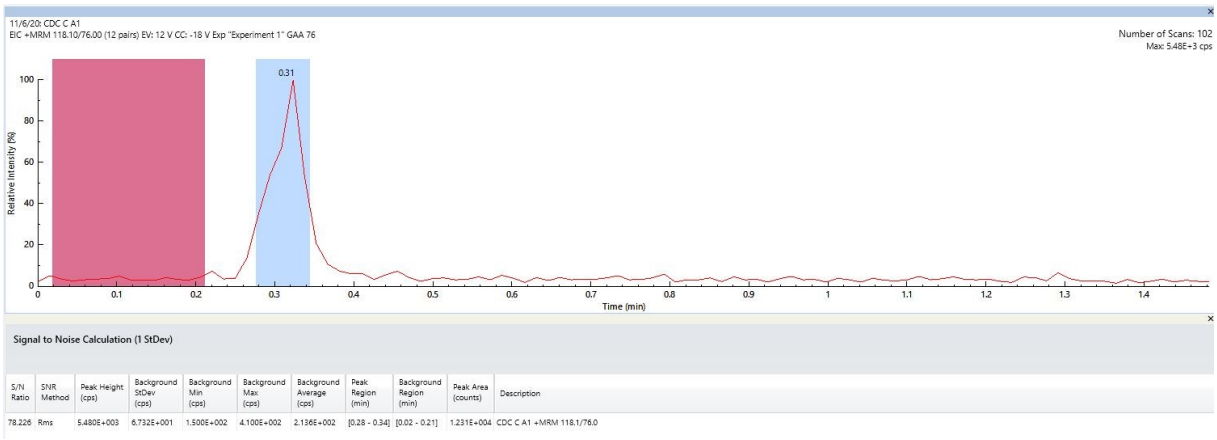


Figure 3.2 Lowest control BSP for GAA (~ 1 µmol/L) for UPLC-MS/MS (S/N ~ 78) measured on a Perkin Elmer Qsight 220.

In contrast, FIA-MS/MS would be possible using a SCIEX API5000 (Figure 3)

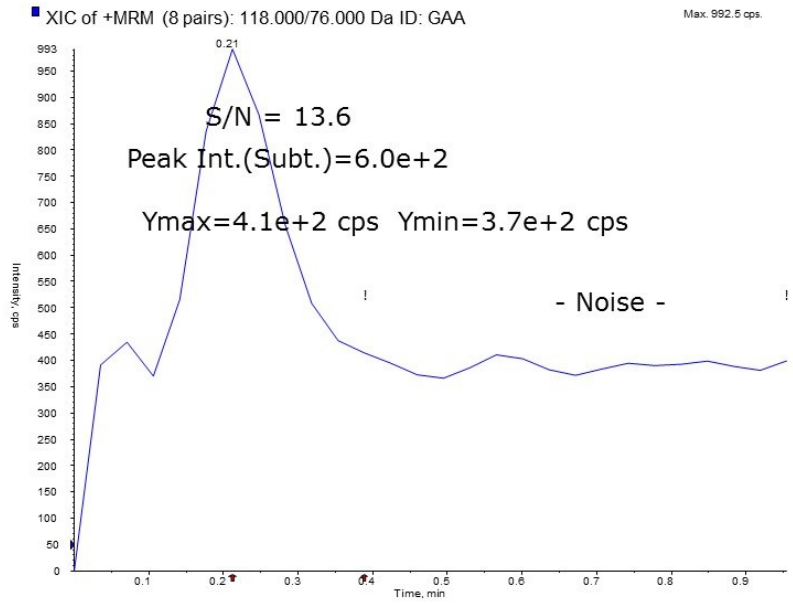


Figure 3.3 Lowest control BSP for GAA (~ 1 µmol/L) for FIA-MS/MS (S/N ~ 14) measured on a SCIEX API5000.

Conclusions:
It is not possible to assess, whether GAA and creatine could be added to the FIA-MS/MS-based (QSight), currently used Neobase II kit. The assessment of an in-house method using LC-MS/MS (Qsight) is possible. A limited test suggested that if a switch would be made to a SCIEX API5000, FIA-MS/MS would be possible for GAA too.

1.1 Optimization of Qsight parameters

The optimal settings for the various parameters of the Qsight were determined for both creatine and GAA. First, product ions for creatine and GAA and their optimal energies were determined (results are listed in paragraph 3.2.4). Since the levels of GAA are near

the quantification limit, the most optimal settings for this compound were used for parameters that cannot be changed within the measurement.

For the determination of the optimal parameter settings a Harvard Apparatus 11 injected 30 µL/min of a 1 µmol/L GAA solution into a flow of 0.5 ml/min of 85% acetonitrile containing 0,1% formic acid.

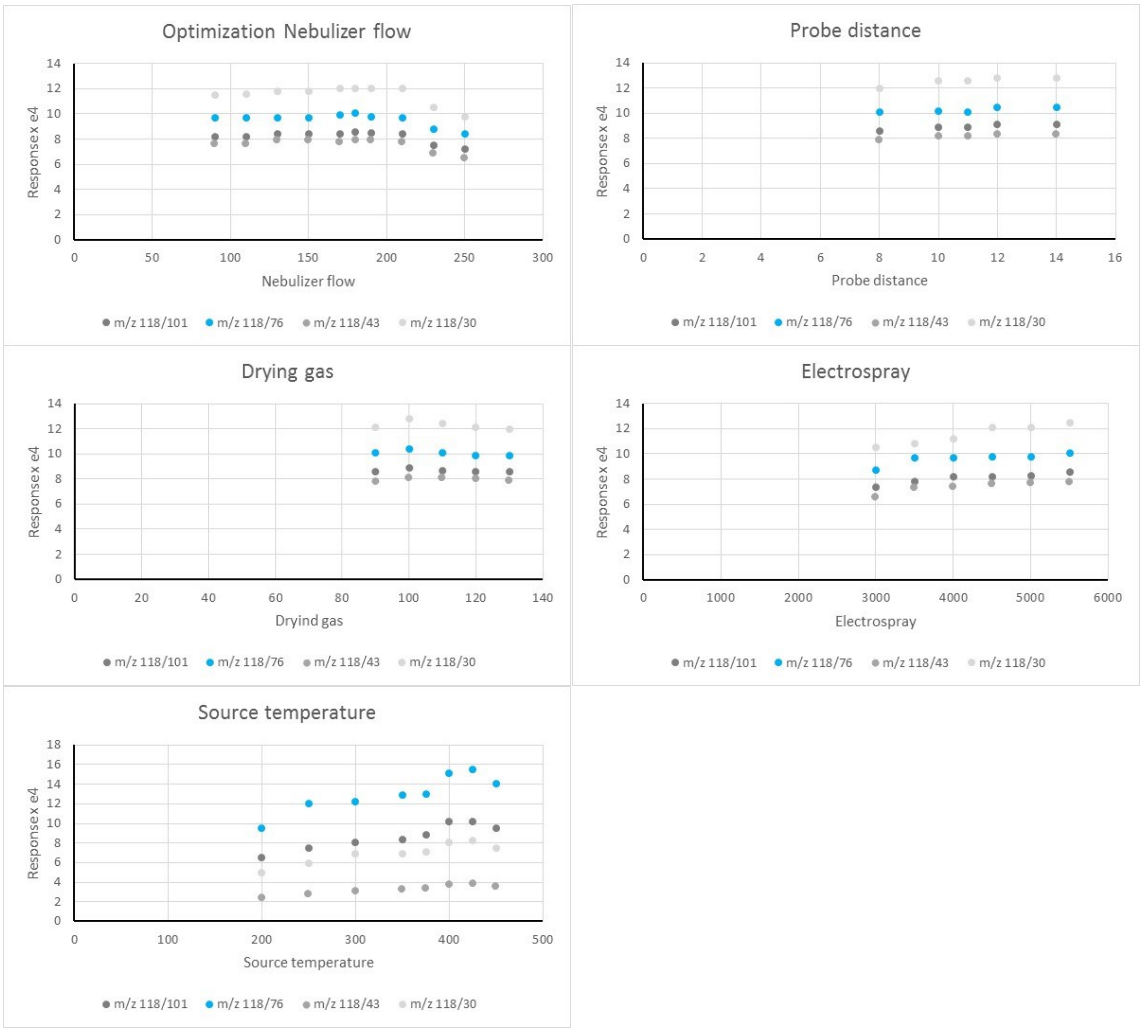


Figure 4 Optimization of parameters of Qsight for various GAA m/z transitions (blue is the quantifier and grey are possible qualifier transitions)

Conclusions:

The most optimal values for the parameters mentioned above are:

Nebulizer flow:	180 L/min
Probe distance:	12 mm
Drying gas:	100 L/min
Electrospray:	5500 V
Source temperature:	425 ºC

All within the recommended values for the flow used (0.5 mL/min).

2 Validation

Since, as was mentioned in paragraph 1, FIA-MS/MS was not possible for GAA, addition of creatine and GAA to the Neobase II was not a valid option hence only the "in-house" method was validated.

2.1 Availability and interchangability of standards from different suppliers

LC-MS often suffers from matrix effects, which can lead either to ion suppression or enhancement affecting the analytical performance. These can be controlled by using isotopically labeled analogues of the compunds measured as internal standards. Calculations of compound concentrations can be done by use of either a calibration curve or a one point calibration for which the internal standard can be used. Both are evaluated throughout this rapport. Since creatine and GAA are not part of any kit, (internal) standards need to be prepared. To ensure continuity of the measurements, these standards need to be either be kept in stock or be interchangable with similar standards of other suppliers.

For the calibration curve method, a wide range of suppliers/sources of unlabeled GAA and creatine are available (Supplementary Table 1-4), but only a limited number of suppliers for isotopically labeled GAA and creatine exists.
Three sources of the unlabeled compounds were compared to each other, including 1 that was already opened >10 years ago (to assess prolonged shelf life). Standards in blood were not available and therefore not tested.

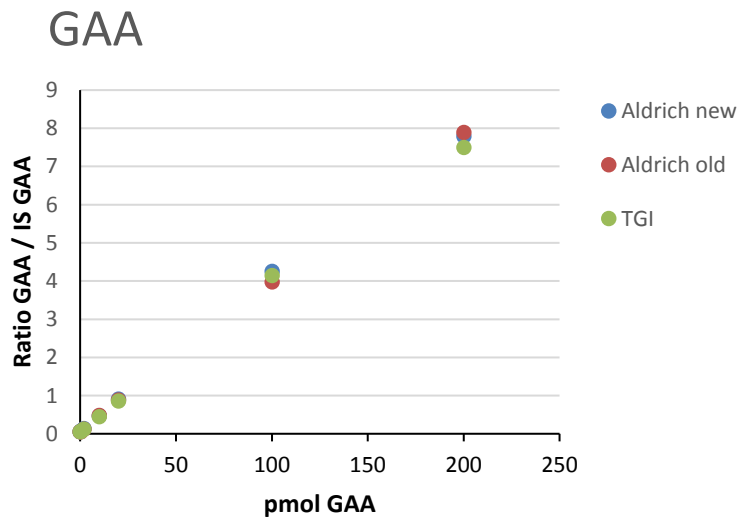


Table 1 Comparison of GAA from different suppliers (CI 95%, SPSS 26).

Comparison	***P***
Aldrich New vs Old	0.472
Aldrich New vs TGI	0.105
Aldrich Old vs TGI	0.555

Figure 5 Comparison of solutions prepared from standards of GAA from different suppliers: Aldrich New (G11608), Aldrich Old (G1160-8; opened 10 years ago), TGI (G0167)

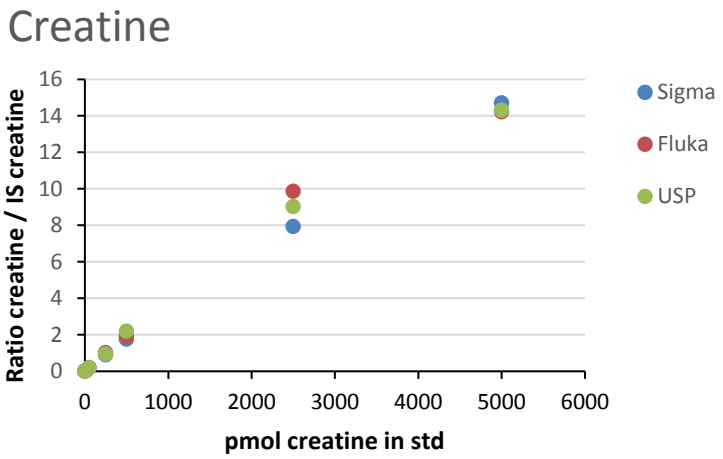


Table 2 Comparison of creatine from different suppliers (CI 95%, SPSS 26).

Comparison	***P***
Sigma & Fluka	0.431
Sigma & USP	0.382
Fluka & USP	0.586

Figure 6 Comparison of solutions prepared from standards of creatine from different suppliers: Sigma (C3630), Fluka (27980; opened 10 years ago, USP reference standard

Conclusions:

There were no statistical differences between the concentrations of the solutions prepared from different suppliers of GAA and creatine. Since GAA and creatine from various suppliers show identical results this will safeguard the continuity of GAMT-D screening. Additional results of the stability assessment are presented in the next paragraph.

2.2 Stability of the standards

IS/standards can be stored either as powder form at room temperature (~22 ºC), when prepared in batches using a freeze dryer or as frozen solutions in the freezer (-20 ºC). The short term stablilty (can standards be kept at room temperature while preparing the bsp samples) and longterm stability of freeze-dried standards and the frozen solution standards was tested. In addition, the pros and cons of the different ways of storing standards are discussed.

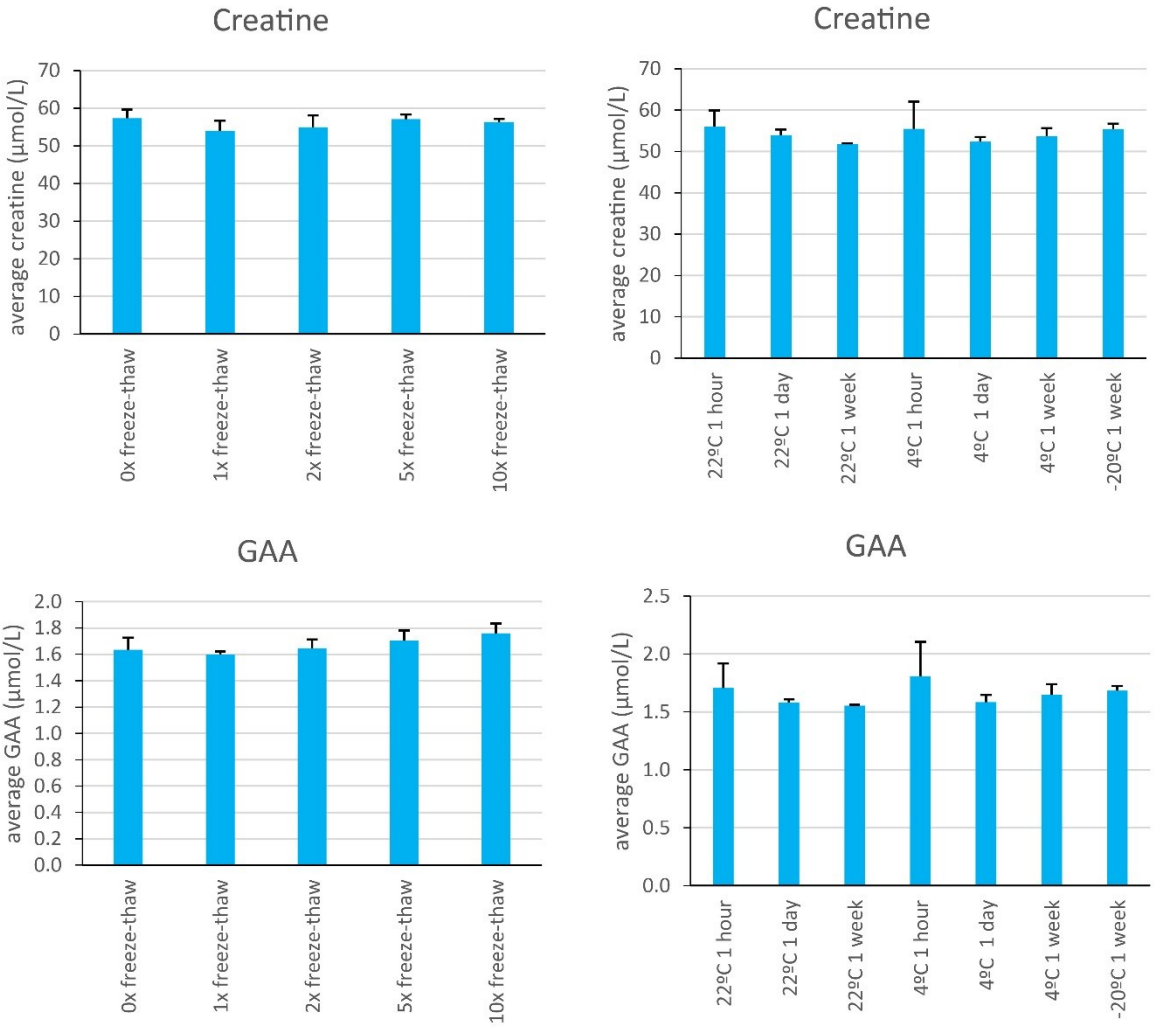


Figure 7 Stability of creatine and GAA during multiple freeze-thaw cycles and during short term storage.

The GAA and creatine concentrations decreased significantly in frozen solutions after one week at room temperature (22ºC) (Table 3).

		P-Value Creatine	P-Value Guanidineacetic acid
Versus 0x freeze-thaw	1x freeze-thaw	0,18	0,57
	2x freeze-thaw	0,35	0,85
	5x freeze-thaw	0,90	0,39
	10x freeze-thaw	0,53	0,13
Versus 1 week -20ºC	22ºC 1 hour	0,79	0,88
	22ºC 1 day	0,27	0,02
	22ºC 1 week	0,01	0,01
	4ºC 1 hour	0,98	0,54
	4ºC 1 day	0,04	0,07
	4ºC 1 week	0,28	0,55

Table 3 Stability of creatine and GAA during multiple freeze-thaw cycles and during short term storage (using SPSS 26).

The same analysis was performed for IS and showed comparable results (not shown). An old standard prepared > 5 years ago was also compared to one that was fresh (Figure 8). No statistical significance (creatine 0.339 and GAA 0.024, n=6, CI 95%) was detected.

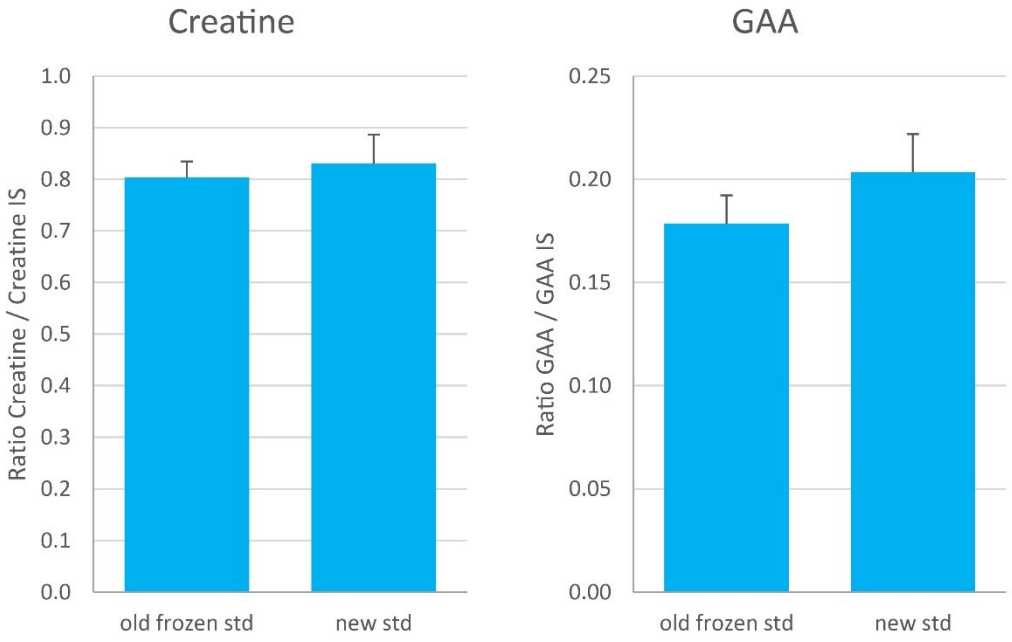


Figure 8 Stability of creatine and GAA in solution during > 5 year storage and multiple freeze-thaw cycles. Using SPSS 26 no statistical significance (creatine<0.339 and GAA<0.024, n=6, CI 95%) was detected.

To test the variability in preparing IS batches, 3 differents IS batches were prepared. One from a frozen solution and 2 from a freeze dried standard (Figure 3.8). No statistical differences were found between the frozen IS solution and the solutions prepared from a freeze dried IS.

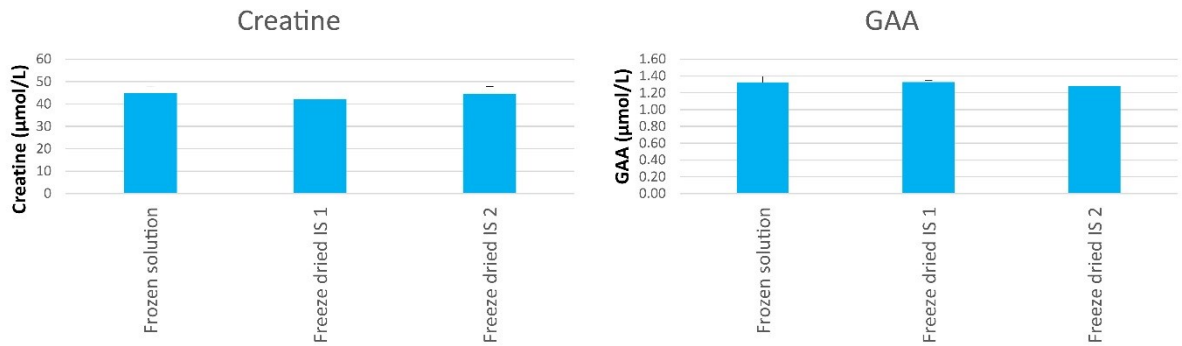


Figure 9 Comparison frozen IS solution to 2 IS solution prepared from freeze dried IS. . Using SPSS 26 no statistical significance (creatine<0.299 and GAA 0.429, n=3, CI 95%) was detected.

Powder standards are stabile at room temperature (~22ºC) for at least 10 years (Figure 5 & 6).

Conclusions:
Powder standards are stabile at room temperature for at least 10 years. Since 3 ng of GAA and 40 ng of creatine are used per sample, storaging 100 mg of each internal standard as back-up would cover more than 2 million samples. This would ensure the continuity of the analysis in the case of delivery issues. In case a calibration will be used 10 mg of unlabeled standard will suffice as back-up.

Since frozen solution standards are stabile at 4ºC for up to 1 week, standards could be potentially prepared only once a week. Internal standards can be prepared in batches either from a frozen solution or from freeze-dried standards. The frozen solution can be stored per daily amount but since multiple freeze-thaw cycles do not affect the stability, storage of larger quantities for multiple days could be possible too.

Based on the results, there are 3 options to store IS/standards:

Storage form	Pros	Cons
Freeze-dried powder	• no freezer neccesary • proven shelf life >10 years	• freeze dryer required
Solution filled out per batch	• easy to use • proven shelf life >5 years	• lot of freezer capacity required
Stock solution that will be used for dilution	• limited freezer capacity required	• extra dilution step • more prone to error because of dilution step • every new solution needs to be tested

The freeze-dried powder would be the preferred option because it is the cheapest and least prone to error. For both option 1 and 2, standards only need to be tested once per batch therefore options would be a good altenative if no freeze dryer is available, stored stock solutions provide the second best option.

2.3 Stability of GAA and creatine in bloodspots

Stabilities of cretine and GAA was investigated in bloodspots taken from 10 volunteers. Variable amounts of GAA was added in five of the samples to gain a wider concentration range. The stabilities were examined for up to week at temperatures of 20 ºC, 25 ºC and 30 ºC mimicking diverse weather conditions, taken into account that the bloodspots are sent by regular post to the laboratory after the collection, and up to 5 weeks of storage, during which the results are to be completed (Figure 10 and 11, Table 4).

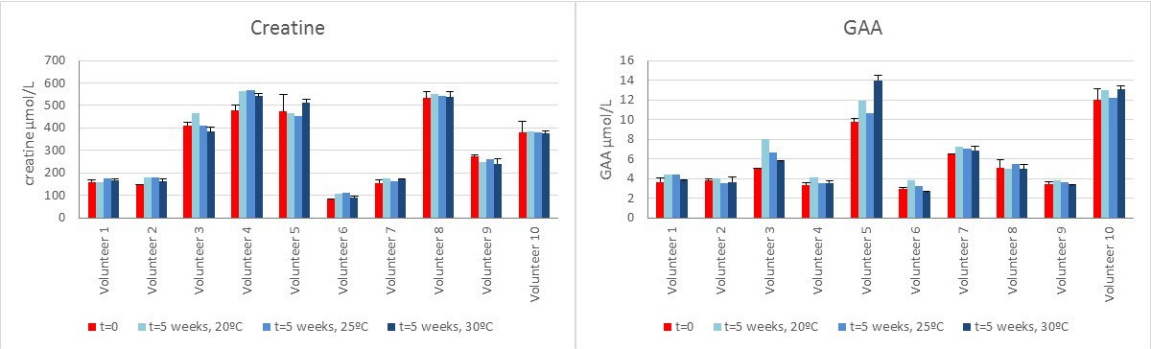


Figure 10 Stability of creatine and GAA in DBS during 5 weeks of storage at 20ºC, 25ºC and 30º (duplicate measurements, bars represent SD).

Using GraphPad Prism 8, Bland-Altman plots were created to assess the effect of storage conditions on the levels of creatine and GAA (Figure 3.10). The bias values for the different conditions are shown in Table 3.4.

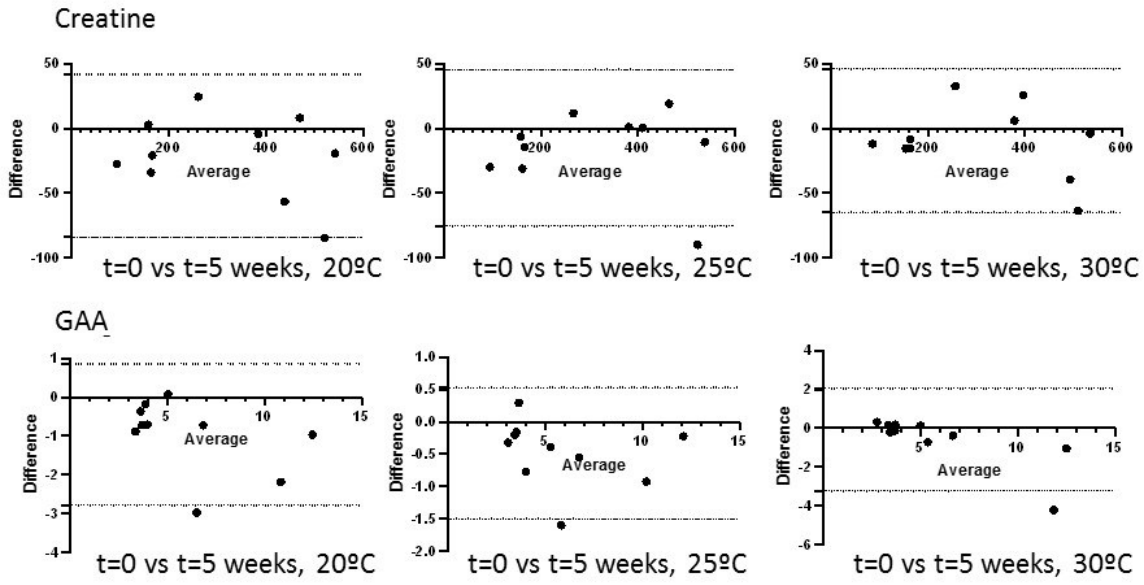


Figure 11 Bland-Altman plots for creatine and GAA (t=0 was compared to the different BSP storage conditions using GraphPad Prism 8, dotted lines represent 95% CI).

	creatine	GAA
t=0 vs t=5 weeks, 20ºC	P<0,066	<0,010
t=0 vs t=5 weeks, 25ºC	P<0,158	<0,016
t=0 vs t=5 weeks, 30ºC	P<0,319	<0,201

Table 4 Effect of storage conditions on the levels of creatine and GAA *(Paired t-test, CI 95%, SPSS 26 was used for analysis).*

Conclusions:

As was observed by Pasquali et al 2014, GAA was stable at room temperature during 5 weeks. Pasquali et al observed a small decrease in creatine, this was however not observed in this study.

2.4 Mass transitions

By using MS/MS molecules can be fragmented. The amount of energy used (collision energy) will determine which fragment predominates. These various mass transitions, with corresponding collision energies were determined for all compounds (Table 5). From all the options the must intense fragment was chosen to quantify the compounds keeping in mind that the fragment needs to be unique too. Since valine has a similar mass as GAA, the fragmens for this compound was also determined to check whether fragment identical to GAA were found. The product spectrum for creatine, GAA and valine are presented in Supplementary Figures 2, 3 and 4. Labeled counterparts of creatine and GAA with 2 different isotopic labels were investigated as well.

Compound	m/z transition	EV	CC L2	CC	Dwell time	Quantifier / Quanlifier
Creatine	132→90	30	-30	-21	5	Quantifier
	132→44	30	-30	-28	5	Qualifier
[D_3]-Creatine	135→93	30	-30	-21	5	Quantifier
	135→47	30	-30	-28	5	Qualifier
[D_5]-Creatine	137→95	30	-30	-21	5	Quantifier
	137→49	30	-30	-28	5	Qualifier
GAA	118→76	12	-24	-18	100	Quantifier
	118→101	12	-24	-17	100	Qualifier
	118→43	12	-24	-30	100	Qualifier
	118→30	12	-24	-30	100	Qualifier
	118→72					Not unique
[$^{13}C_2$]-GAA	120→103	12	-24	-18	100	Qualifier
	120→78	12	-24	-17	100	Quantifier
	120→43	12	-24	-30	100	Qualifier
	120→31	12	-24	-30	100	Qualifier
[D_2]-GAA	120→103	12	-24	-18	100	Qualifier
	120→78	12	-24	-17	100	Quantifier
	120→43	12	-24	-30	100	Qualifier
	120→32	12	-24	-30	100	Qualifier
Valine	118→72					Not unique

Table 5 Mass transitions and fragmentation parameters for creatine, GAA and valine (Qsight, Perkin Elmer).

Conclusions:

For creatine, the most intense m/z transition 132→90 will be used as quantifier ion. For GAA, m/z transition 118→76 will be used as quantifier ion. The most intense product ion found for GAA, m/z transition 118→72 cannot be used, since Valine and GAA share this m/z transition. 118→72, and hence, elevations in GAA concentrations could remain unnoticed using this m/z transition (valine expected concentraions are around 100 µmol/L and for GAA patients around 20 µmol/L). The fragment 76 results from the loss of the guanidine group from GAA. Isomers like valine, norvaline and betaine will not contribute to signal of this fragment, since they do not have a guanidine group. The labeled

counterparts of GAA and creatine confirmed the unique fragments found. Corresponding m/z transitions were chosen for the labeled counterparts of GAA and creatine.

2.5 Mass spectrometric purity of standards

To ensure that cross-over between labeled and unlabeled compounds is not possible, the purity of the various standards was assessed, including an additional IS.

For creatine, only [D_3]-creatine contributed to signal of [D_5]-creatine (natural 13C isotope of [D_3]-creatine).

	unlabeled		D_3		D_5	
	quantifier	qualifier	quantifier	qualifier	quantifier	qualifier
m/z	132→90	132→44	135→93	135→47	137→95	137→49
Creatine	-	-	0,00%	0,00%	0,00%	0,00%
[D3]-Creatine	0,01%	0,00%	-	-	2,62%	0,11%
[D5]Creatine	0,00%	0,05%	0,00%	0,00%	-	-

Table 6 Contribution of creatine, IS [D3]-creatine and alternative IS [D5]-creatine to each other's signals.

For GAA, [$^{13}C_2$]-GAA and [D_2]-GAA contributed to each other signals.

	unlabeled		$^{13}C_2$		D_2	
	quantifier	qualifier	quantifier	qualitier	quantifier	qualifer
m/z	118→76	118→101	120→78	120→103	120→78	120→103
GAA	-	-	0,00%	0,00%	0,00%	0,00%
[13C2]-GAA	0,01%	0,01%	-	-	-	1,85%
[D2]-GAA	0,16%	0,12%	-	2,77%	-	-

Table 7 Contribution of GAA, IS [13C2]-GAA and alternative IS [D2]-GAA to each other's

The internal standards did not significantly contribute to the signals of unlabeled standards (minor detected contribution of [D_2]-GAA to unlabeled guanidinoacetate, 0.16%, is not considered significant).

Conclusions:
All the labeled compounds which were measured are applicable as internal standards. The observations of the contributions between some labeled internal standards are not practically relevant, since these standards not used simultaneously.

2.6 Fidelity of quantifier fragment

Qualifier fragments are used to ensure that the response for the compound is not elevated due to a contribution of other compounds sharing the same m/z transition. An applicable qualifier fragment shows a ratio of ~1 (±10%) of the peak area in comparison to the quantifier fragment. A ratio larger than 1 indicates that other compounds contribute to the m/z signal.

		Ratio quanitifier/qualifier	Average	Std. Dev.	CV %	n
Creatine	Std	132→90 / 132→44	1,00	0,06	6	57
	IS	135→93 / 135→49				
GAA	Std	118→76 / 118→101	1,04	0,15	14	57
	IS	120→78 / 120→103				
	Std	118→76 / 118→43	2,29	0,17	7	57
	IS	118→76 / 118→43				
	Std	118→76 / 118→30	1,66	0,19	11	57
	IS	118→76 / 118→31				

Table 8 Fidelity of the quantifier fragment (ratio should be ~1±10%) for creatine and GAA for standards in water.

For creatine the qualifier/quantifier fragment ratio was 1.00 (acceptable 0.9 – 1.1) with a and CV of 6% which makes m/z transition 132→90 an acceptable qunatifier mass transition. For GAA the ratio was 1.04 which makes 118→76 an acceptable quantifier mass transition but the CV was 15%. This is mainly caused by the fact that the intensity for the m/z 118→101 is realy low which leaves GAA without an acceptable qualifier mass transition.

Conclusions:
In water, the m/z transition 132→/44 for standard creatine and 118→101 for standard GAA are applicable to monitor compound fidelity. Unfortunately in BSP for GAA the qualifier fragment is below the detection limit for controls. The creatine qualifier can be used to check fidelity of the creatine peak (data not shown).

2.7 Linearity

Linearity for both creatine and GAA were determined in water and BSP (by spiking known amounts of creatine and GAA to blood) in triplicate. Residuals were calculated to assess linearity of the response. Calibration curve and one point calculations can only be used under the condition that the measured concentration fall within in the linear range.

In water, creatine showed linearity from 10-3000 pmol (for a 10 µL injection), which translates to 1.85-566 µmol/L in BSP (required range, 138-831 µmol/L).

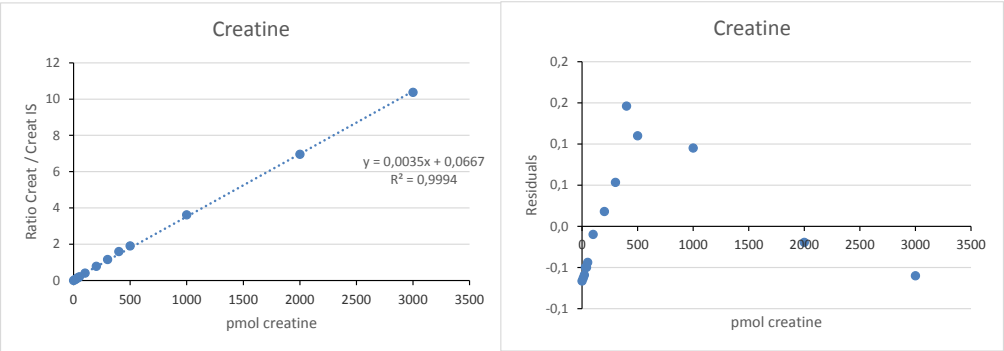


Figure 12 Linearity for creatine in water (for a 10 µL injection).

In water GAA showed linearity from 0.4-160 pmol (for a 10 µL injection), which translates to 0.07 -29.6 µmol/L in BSP (range necessary 0.5-28.7 µmol/L).

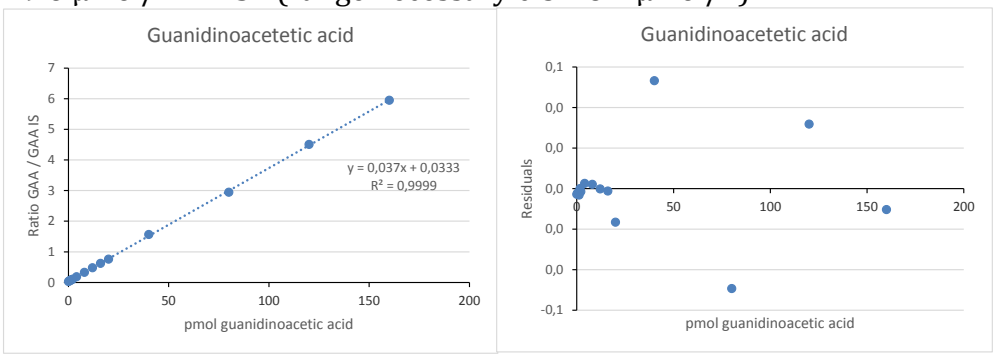


Figure 13 Linearity for GAA in water (for a 10 µL injection).

In BSP, creatine showed linearity in 1/8 inch BSP from 50-5000 pmol (for a 10 µL injection), which translates to 16.7-1667 µmol/L in BSP (required range, 138-877 µmol/L).

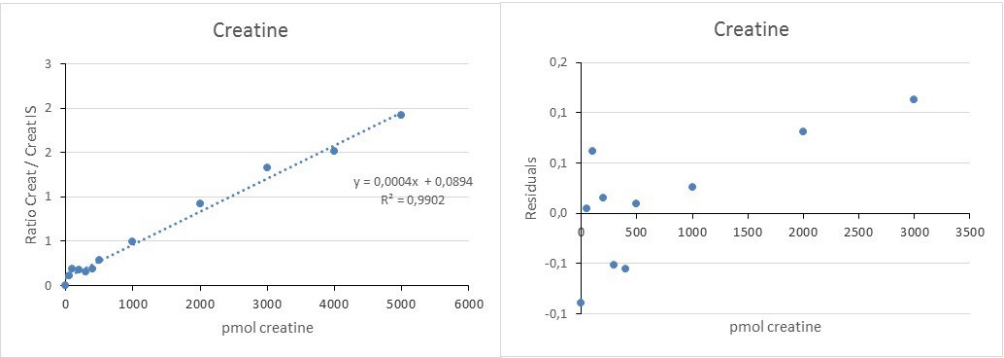


Figure 14 Linearity for creatine in 1/8 inch BSP (for a 10 µL injection).

GAA showed linearity in 1/8 inch BSP from 0.4-160 pmol (for a 10 µL injection), which translates to 0.12 -44.4 µmol/L in BSP (required range, 0.5-28.7 µmol/L).

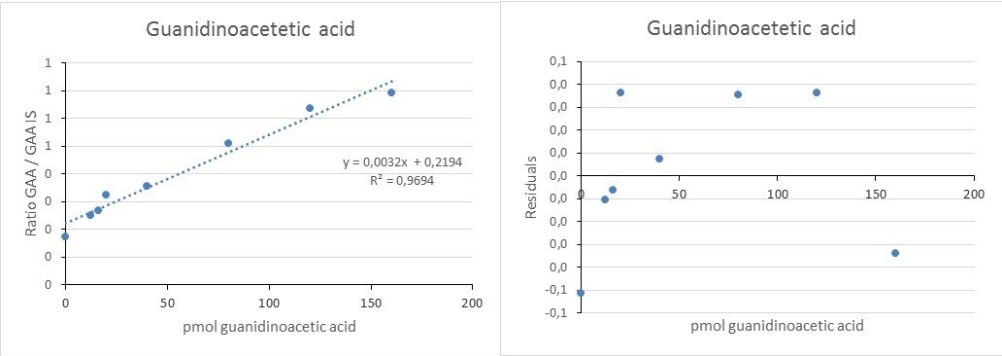


Figure 15 Linearity for GAA in 1/8 inch BSP (for a 10 µL injection).

	Creatine			GAA		
	Average	Std Dev	CV %	Average	Std Dev	CV %
LOQ	48,6	1,8	4%	0,73	0,05	6,5%

Table 3.9 LOQ parameters for creatine and GAA in 1/8 inch BSP (n=10).

Conclusions:

Linearity for creatine in water is not adequate to measure the BSP (832 µmol/L is necessary but only until 566 µmol/L linear. If the measurement with a calibration curve is chosen, only 5 µL instead of 10 µL of the highest to 2 standards needs to be injected. Since mass suppression in BSP leads to a higher linearity range, the measurements are expected to fall within the linearity range, so for bsp samples there is no linearity issue.

2.8 Quantitation limit

Dilutions of the BSP controls provided by CDC (Centers for Disease Control and Prevention) were used to assess the quantification limit (S/N >10) of 1/8 inch BSP. The lowest sample that was above the quantification limit was measured 10 times to ensure S/N was >10 (determined by Qsight software). Subsequently, the CV of this measurement was determined.

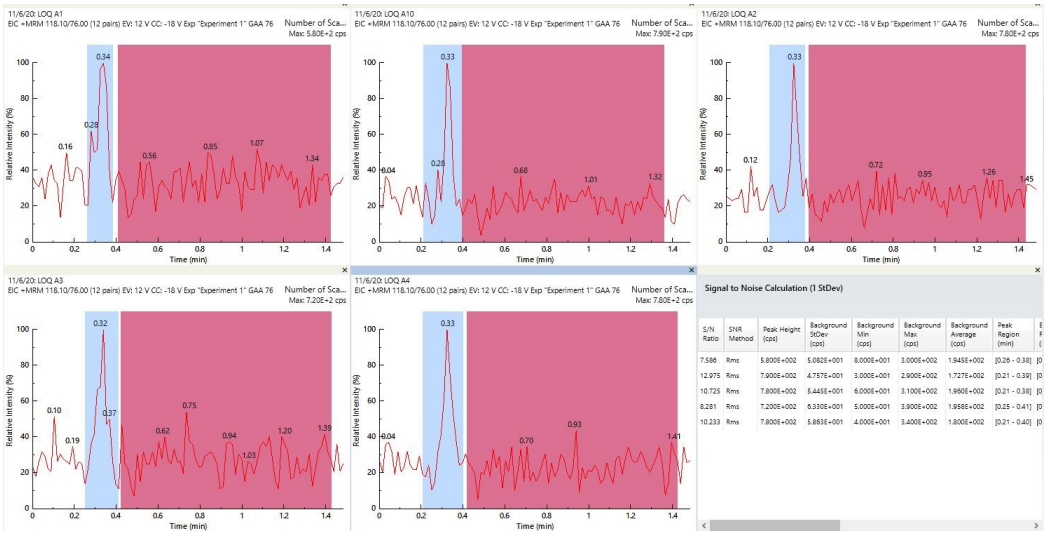


Figure 16 LOQ of GAA in 1/8 inch BSP (n=5, average S/N >10 for 0.73 µmol/L).

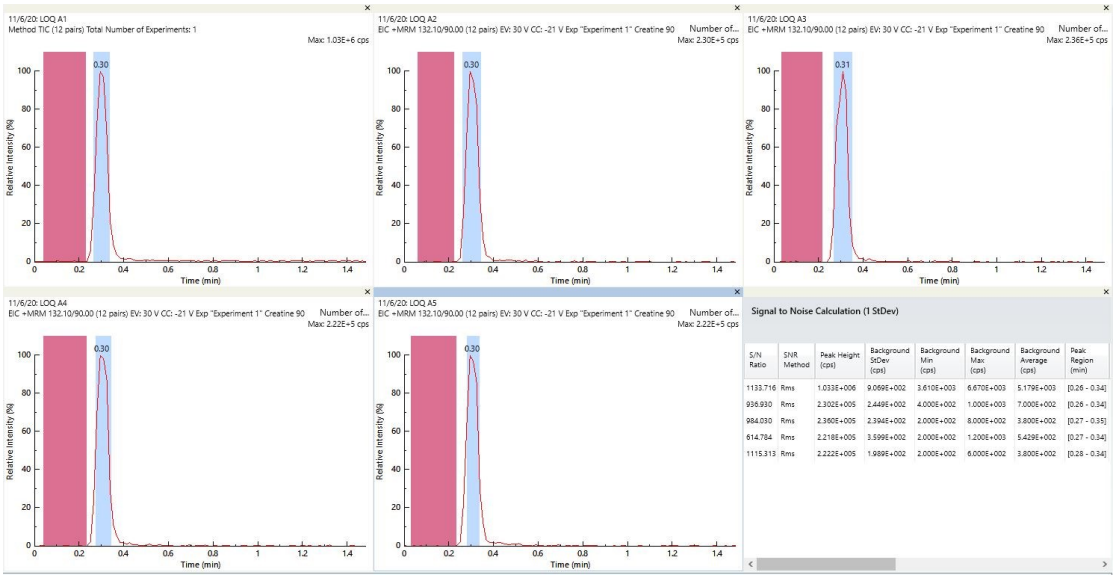


Figure 17 LOQ of creatine in 1/8 inch BSP (n=5, average S/N >1000 for 48.6 µmol/L). BSP could not be diluted more within compromizing the matrix effect.

Conclusions:

For creatine the actual detection limit is much lower than the value shown but in order to adequately determine the LOQ for creatine, bloodspots need to be diluted to such an extent that the matrix is no longer comparable to actual bloodspots. The LOQ for creatine is <48.6 µmol/L. This LOQ is adequate since expected concentrations are 138-877 µmol/L for controls and 208–392 µmol/L for patients (Pasquali et al, 2014). The LOQ for GAA <0.73 µmol/L. This LOQ makes detection of GAA for controls a bit on the limit (expected concentrations are 0.5-10.4 µmol/L) but adequate to detect patients (expected concentrations 12.1–28.7 µmol/L, Pasquali et al, 2014), which makes the method suitable for GAMT-D patient detection.

2.9 Recovery

To test whether creatine and GAA are adequately extracted from the BSP, variable amounts of creatine and GAA were added to the blood samples from a volunteer (see Table 3.10). BSPs were prepared and following measurement, the recovered amount of creatine and GAA was calculated.

Creatine		GAA	
Added pmol	Recovery %	Added pmol	Recovery %
75	44	3	44
150	96	6	88
300	96	12	87
450	104	18	89
600	97	24	85
750	106	30	83

Table 10 Recovery % of creatine and GAA from bloodspots (10 pmol ~ 3 µmol/L in BSP).

Except for the lowest addition (the added amount approximately 10% of total concentration), for both creatine and GAA the method showed good extraction efficiency using the Neobase II extraction solution. Other extraction solution (for example 85% acetonitril, 0,1 % formic acid) were investigated as well but show for GAA less extraction efficiency (50-60%).

Conclusions:
The extraction efficiency from BSPs for both creatine and GAA is adequate using the Neobase II extraction solution.

2.10 Carry-over

When high samples are injected you run the risk of contaminating the next sample. To assess carry-over of high samples 10 µL of a high standard (5000 pmol creatine and 200 pmol GAA) and a low standard (12.5 pmol creatine and 0,5 pmol GAA) are injected in the sequence L1, L2, L3, H1, H2, L4, H3, H4, L5, L6, L7, L8, H5, H6, L9, H7, H8, L10, H9, H10 and L11. The same is done for the highest CDC BSP control (D ~ 630 µmol/L creatine and 20 µmol/L GAA and the lowest CDC BSP control (A ~ 185 µmol/L creatine and 1 µmol/L GAA).

Carry-over is determined by subtracting the average of L2,L3, L6, L7 and L8 from the average of L4, L5, L9, L10 and L11. Subsequently, the percentage of carry-over of the average high standard or high CDC BSP control is calculated. The results are in Table 11.

		Carry-over	% of average
BSP	Creatine	-0,061	-0,8%
Std	Creatine	0,004	0,0%
BSP	GAA	0,004	0,3%
Std	GAA	0,000	0,0%

Table 11 Carry-over of high standards (5000 pmol creatine and 200 pmol GAA) or BSP (~630 µmol/L creatine and 20 µmol/L GAA) to following samples for creatine and GAA.

For both matrices (water and BSP), there was less tha 1% carry-over for creatine and GAA.

Conclusions:
High concentration samples will not contribute to the result of the following sample which makes the method adequate for measurement of creatine and GAA within the necessasry concentration range.

2.11 Drift

CDC profiency samples concentration were followed over time to determine drift of the the mehod

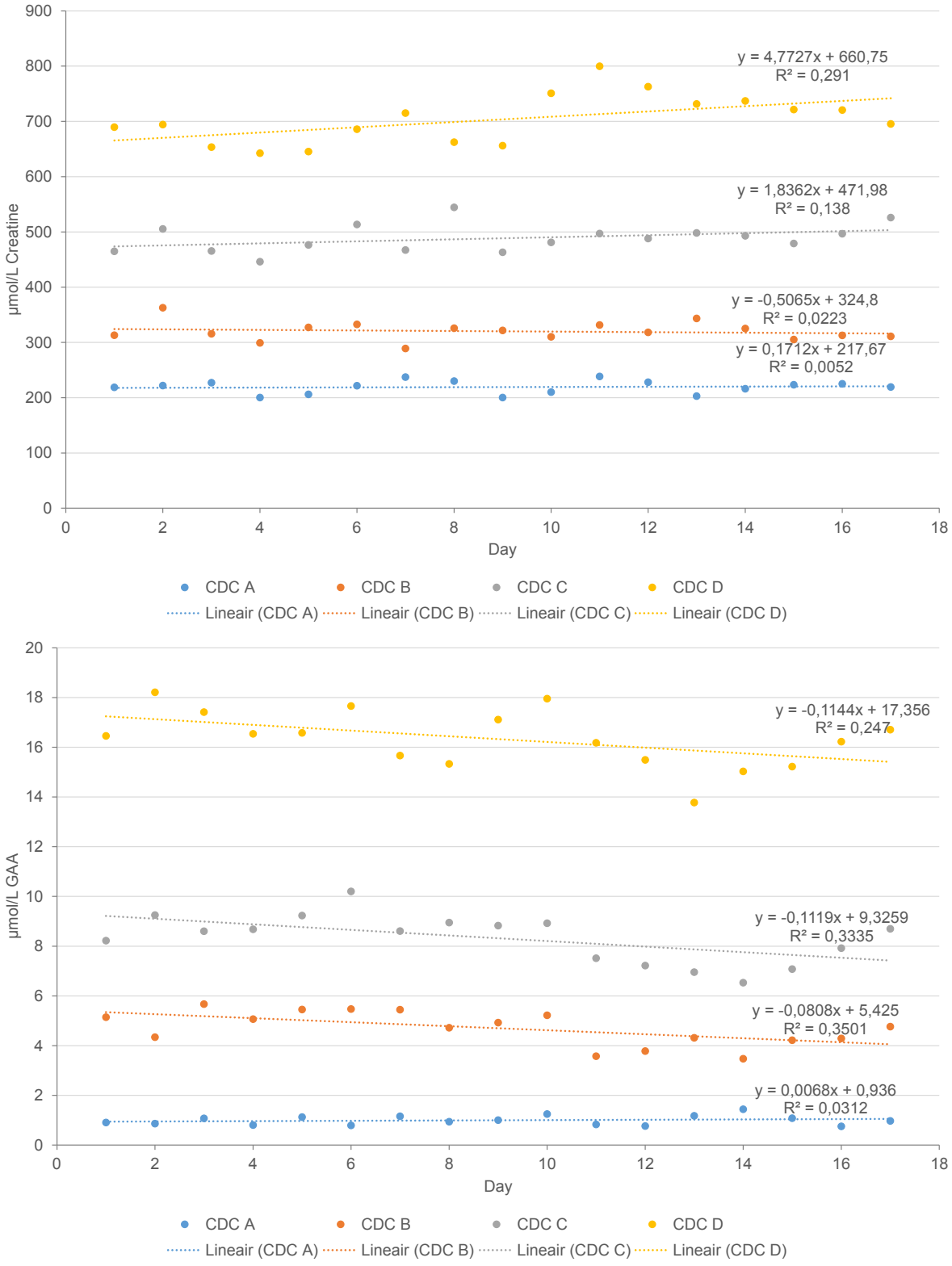


Figure 18 Drift in creatine and GAA concentration evaluation in CDC profiency samples.

Conclusions:
Drift was within the variation of the method.

2.12 Intra and inter-assay variation

As was mentioned in paragraph 2.2, during this validation 2 methods for the determination of the concentrations of creatine and GAA were evaluated, a calibration curve and calculation using the internal standard. In this paragraph, the intra- and inter-assay variation using CDC profiency samples were assessed for both methods and their performance characteristics were compared. CDC profiency samples were used because they offer a concentration range for both analytes. The intra- and inter-assay variation for newborn screening samples were also determined using 2 random samples.

Intra-assay variation

Profidiency sample CDC	This assay			Interlab	
	Avg µmol/L	Std Dev µmol/L	CV %	Avg µmol/L	Std Dev µmol/L
Creatine					
CDC A lot1915	228,5	14,6	6%	185,5	50,2
CDC B lot1915	327,2	16,4	5%	272,7	55,6
CDC C lot1915	486,6	27,8	6%	426,8	90,9
CDC D lot1915	722,1	48,9	7%	630,6	107,2
GAA					
CDC A lot1915	1,14	0,17	15%	1,36	0,61
CDC B lot1915	6,09	0,43	7%	5,87	1,84
CDC C lot1915	9,67	0,47	5%	10,06	3,3
CDC D lot1915	19,27	0,97	5%	19,58	6,83

Table 12 Intra-assay variation in 1/8 inch BSP quantified using a calibration curve (interlab results for these proficiency samples are listed on the right).

Profidiency sample CDC	This assay			Interlab	
	Avg µmol/L	Std Dev µmol/L	CV %	Avg µmol/L	Std Dev µmol/L
Creatine					
CDC A lot1915	218,9	13,9	6%	185,5	50,2
CDC B lot1915	313,0	15,7	5%	272,7	55,6
CDC C lot1915	464,9	26,5	6%	426,8	90,9
CDC D lot1915	689,6	46,6	7%	630,6	107,2
GAA					
CDC A lot1915	0,91	0,15	16%	1,36	0,61
CDC B lot1915	5,15	0,37	7%	5,87	1,84
CDC C lot1915	8,22	0,41	5%	10,06	3,3
CDC D lot1915	16,46	0,83	5%	19,58	6,83

Table 13 Intra-assay variation in 1/8 inch BSP quantified using the IS (n=16, interlab results for these proficiency samples are listed on the right).

Inter-assay variation

Profidiency sample CDC	This assay			Interlab	
	Avg µmol/L	Std Dev µmol/L	CV %	Avg µmol/L	Std Dev µmol/L
Creatine					
CDC A lot1915	223,7	16,1	7%	185,5	50,2
CDC B lot1915	328,0	17,4	5%	272,7	55,6
CDC C lot1915	491,1	13,6	3%	426,8	90,9
CDC D lot1915	697,1	29,2	4%	630,6	107,2
GAA					
CDC A lot1915	0,98	0,32	33%	1,36	0,61
CDC B lot1915	6,12	0,53	9%	5,87	1,84
CDC C lot1915	10,65	0,69	6%	10,06	3,3
CDC D lot1915	20,24	1,01	5%	19,58	6,83

Table 14 Inter-assay variation in 1/8 inch BSP quantified using a calibration curve (n=10, interlab results for these proficiency samples are listed on the right).

Profidiency sample CDC	This assay			Interlab	
	Avg µmol/L	Std Dev µmol/L	CV %	Avg µmol/L	Std Dev µmol/L
Creatine					
CDC A lot1915	220,4	12,2	6%	185,5	50,2
CDC B lot1915	320,6	22,5	7%	272,7	55,6
CDC C lot1915	485,4	32,6	7%	426,8	90,9
CDC D lot1915	673,5	26,3	4%	630,6	107,2
GAA					
CDC A lot1915	0,96	0,14	15%	1,36	0,61
CDC B lot1915	5,16	0,45	9%	5,87	1,84
CDC C lot1915	8,97	0,61	7%	10,06	3,30
CDC D lot1915	16,73	0,98	6%	19,58	6,83

Table 15 Inter-assay variation in 1/8 inch BSP quantified using the IS (interlab results for these proficiency samples are listed on the right).

Both the intra- and inter-assay variation all results are within 1SD of the interlab results. For the intra-assay, the method of quantification has no influence on the variation but has effect on the accuracy of the results. Results calculated using the calibration curve result in higher levels for both creatine as GAA, probably due to linearity issues with the higher standards in water. Quantification using IS has for the inter-assay a beneficial effect on the variation of the lowest CDC sample for GAA.

Intra- and inter-assay variation newborn screening samples

Newborn screening	intra-assay			inter-assay		
	Avg µmol/L	Std Dev µmol/L	CV %	Avg µmol/L	Std Dev µmol/L	CV %
Creatine						
Sample 1	200,2	22,6	11%			
Sample 2	280,4	22,2	8%	264,2	11,8	4%
GAA						
Sample1	1,42	0,13	9%			
Sample 2	1,25	0,29	23%	1,26	0,29	23%

Table 16 Intra- and inter-assay variation (n=10 & n=7) in random 1/8 inch newborn screening BSP.

Variation in random newborn screening BSP is comparable to the lowest profiency sample.

Conclusions:

All CV results are <9% except for the lowest CDC sample for GAA, which is to be expected since it is near the LOQ for this method. Since the lowest GAA concentration reported is 4.93 µmol/L (Pasquali) well over this range, the method is adequate to detect GAMT-D patients. The method can be used for the measurement of creatine and GAA in 1/8 inch BSPs. Using the method where the concentration is calculated using the internal standard would be preferrable since it is more straightforward and omits the need for standard preparation and measurement.

2.13 Robustness

Extraction efficiency is one of the main determinant of the robustness of the in-house method, therefore 3 batch numbers of extraction solution were measured in triplicate and compared.

	Creatine		GAA	
	Avg	Std Dev	Avg	Std Dev
Proficiency sample CDC B				
Lot 1	337,6	25,9	6,11	0,50
Lot 2	321,6	32,7	8,23	0,78
Lot 3	308,1	12,8	6,61	1,84
Sig. (one way Anova)	0,412		0,069	
Proficiency sample CDC C				
Lot 1	502,0	23,7	10,01	0,64
Lot 2	466,2	25,6	14,12	2,08
Lot 3	452,3	12,3	15,19	0,96
Sig. (one way Anova)	0,155		0,008	

Table 17 Variation between 3 different batch numbers of extraction solution (using SPSS 26).

Conclusions:
Extraction efficiency variation was within the variation of the method (see paragraph 2.12).

2.14 Alternative IS

Since [$^{13}C_2$]-GAA (AmsterdamUMC) is available from a limited number of suppliers, an alternative IS [D_2]-GAA (CDN isotopes) was tested to assess whether this could be used as alternative IS. The results of 4 different levels of proficiency samples were compared.

No statistical differences were found between the concentratios measured using the different labeled internal standard (Table 17)

Profidiency sample CDC	[13C2]-GAA			[D2]-GAA			
	Avg µmol/L	Std Dev µmol/L	CV %	Avg µmol/L	Std Dev µmol/L	CV %	Sig.
CDC A lot1915	0,91	0,15	0,16	1,01	0,23	0,23	0,534
CDC B lot1915	5,15	0,37	0,07	5,56	0,38	0,07	0,208
CDC C lot1915	8,22	0,41	0,05	9,24	0,40	0,04	0,030
CDC D lot1915	16,46	0,83	0,05	18,36	1,36	0,07	0,128

Table 18 Variation between using [$^{13}C_2$]-GAA (purity >99%) and [D_2]-GAA (purity>98%) as internal standard (n=3, using SPSS 26)

Conclusions:
[D_2]-GAA can be used as alternative IS for [$^{13}C_2$]-GAA.

2.15 Proficiency samples

The Centers for disease control and prevention (CDC) offer laboratories a quality ensurance program with 4 level creatine/GAA BSP proficiency samples (https://www.cdc.gov/labstandards/nsqap_resources.html). These samples were used for validation of this newly developed method.

2.16 Overall conclusion validation

During optimization FIA-MS/MS of 1/8 inch BSP, using the same protocol that is currently used in the Dutch newborn screening turned out to be not feasible using the Perkin Elmer QSight 220 mass spectrometer due to a combination of severe suppression of the GAA signal by the BSP matrix and the low concentrations of this compound. Therefor only the in-house method was validated. If another for the Dutch newborn screening wouuld be purchased FIA-MS/MS is possible. For the SCIEX API5000 the LOQ for GAA was adequate enough for measurement.

In order to identify GAMT-D patients accurately in newborn screening BSP samples, the method has to comply with the criteria discussed previously:

Stability standards	(internal) standards are during sample preparation and storage (powder recommended)
Stability in BSP	creatine and GAA are stable in BSP for the duration of the screening timeframe
Mass transition	Creatine and GAA have unique mass fragments to be measured
Isotopic purity standards	Standards are isotopically pure
Qualifier fragment	Creatine has a useful alternative fragment that can be used to ensure fidelity of the result of the quantifier fragment. For GAA the intensity for this fragment is too low for the low reference concentrations.
Linearity	Creatine and GAA displayed a linear response in the expected range for creatine and GAA found in literature
LOQ	For creatine the LOQ was well below the expected range. For GAA the LOQ was <0.73 µmol/L which makes it a bit on the limit for controls but adequate for patients. An added pro of this LOQ is that patients with AGAT defiency, which display low GAA, will not be found with this method.
Recovery	Extraction efficiency from BSP was adequate for creatine (100%0 anf GAA (87%).
Carry-over	The highest expected concentrations did not results in elevations in the following sample
Drift	No drift in the concentrations in time was found.
Accuracy	Concentrations of proficiency samples were within 1SD of other laboratories
Precision	All CV results are <9% except for the lowest CDC sample for GAA, which is to be expected since it is near the LOQ for this method.
Robustness	Extraction solution with different lotnumbers did not result in deviant concentrations.
Profiency samples	Available from Centers for disease control and prevention.

(Internal) standards are available from various suppliers and are interchangable.

Since all parameters fall within the preset criteria, the designed method is adequate to detect GAMT-D patients in newborn screening samples.

3 Reference values & cut-off values

For the determination of the reference values and subsequent cut-off values, 469 bloodspot punches from the current screening where anonymized and divided into 4 groups:

- Group 1: Female, a terme
- Group 2: Male, a terme

- Group 3: Female, premature
- Group 4: Male, premature

(premature < 36 weeks of pregnancy)

3.1 Comparision between groups

Subsequently creatine, GAA and the ratio GAA/creatine waere measured and the results of the groups were compare to each other (Figure 19).

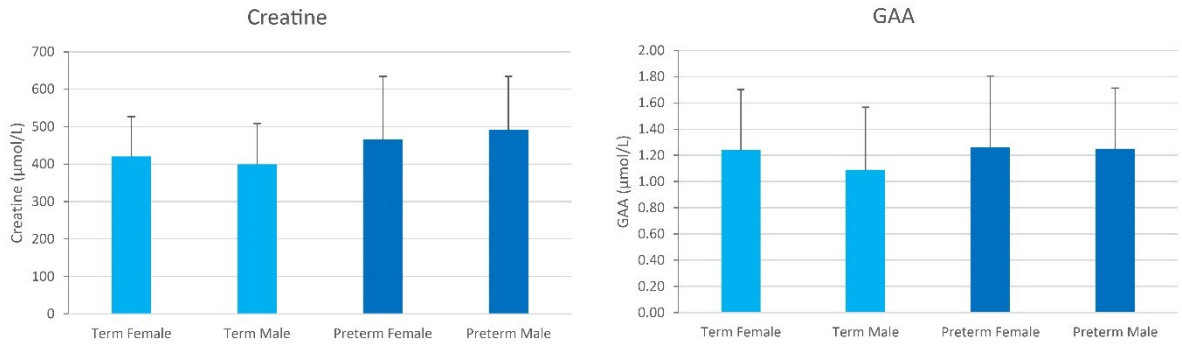


Figure 19 Average Creatine and GAA levels in BSP of a terme and premature children (bar represents SD, n~120 per group)

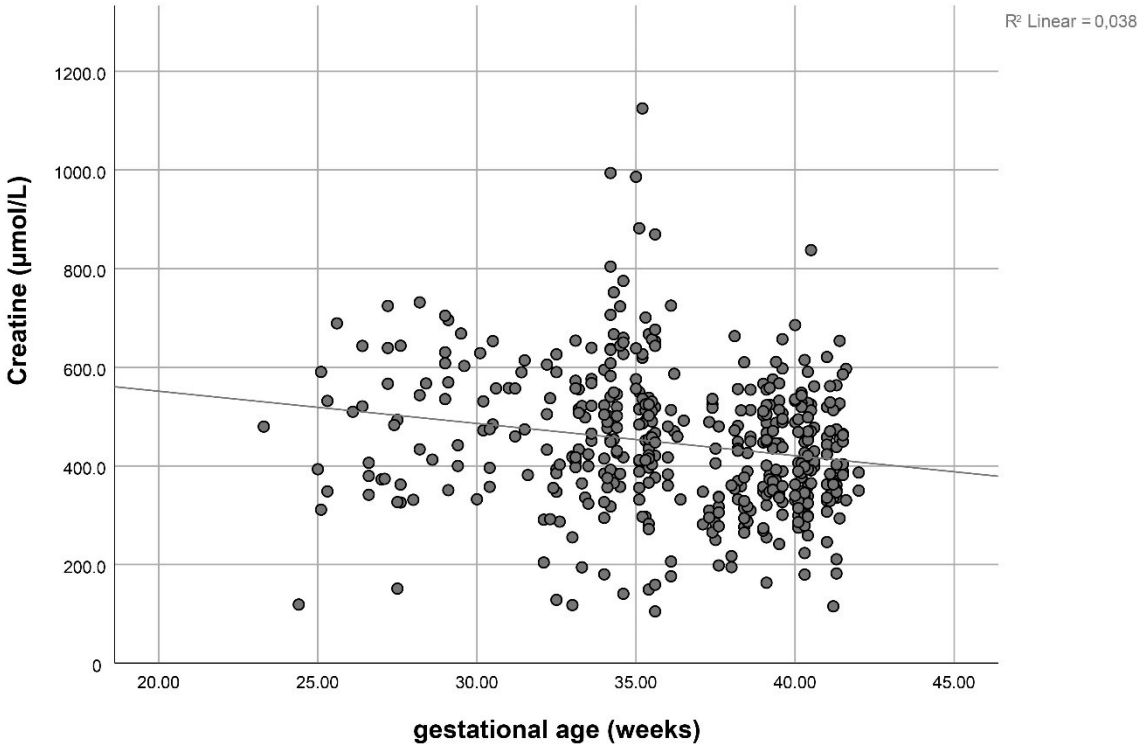
Creatine and GAA were significantly higher in premature males in comparison to a terme males. There were no differences found between the other groups.

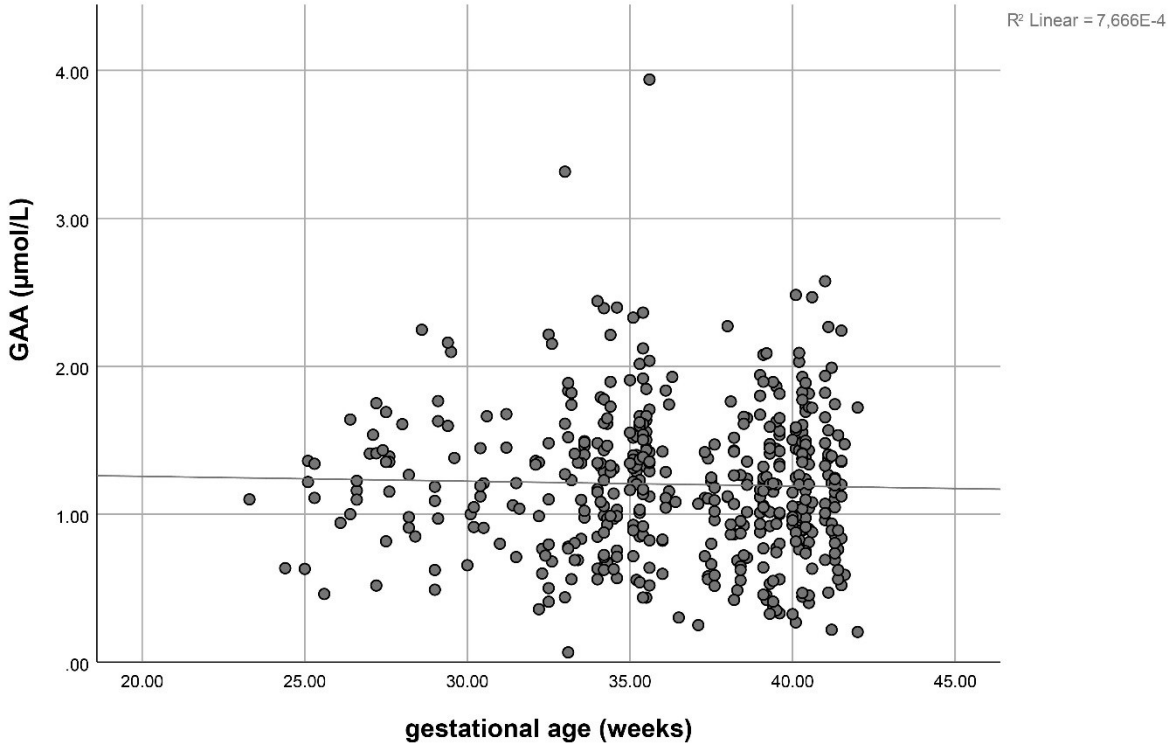
	Sig. (2-tailed)		
	creatine	GAA	GAA/Creatine
f a terme versus m a terme	0.133	0.015	0.233
f a terme versus f prematuur	0.020	0.811	0.554
f prematuur versus m prematuur	0.180	0.976	0.317
m a terme versus m prematuur	0.000	0.008	0.640

Table 19 Differences between creatine and GAA and GAA/creatine levels between the various groups (Independent Student T-test, SPSS).

3.2 Pregnancy duration

For all samples the duration of the pregnancy was known. Therefore scatterplots where made to investigate if a trend of creatine and GAA levels and duration of pregnancy was present.


R² Linear = 0,038
Creatine (µmol/L)
1200.0
1000.0
800.0
600.0
400.0
200.0
0
20.00
25.00
30.00
35.00
40.00
45.00
gestational age (weeks)


R² Linear = 7,666E-4
GAA (µmol/L)
4.00
3.00
2.00
1.00
.00
20.00
25.00
30.00
35.00
40.00
45.00
gestational age (weeks)

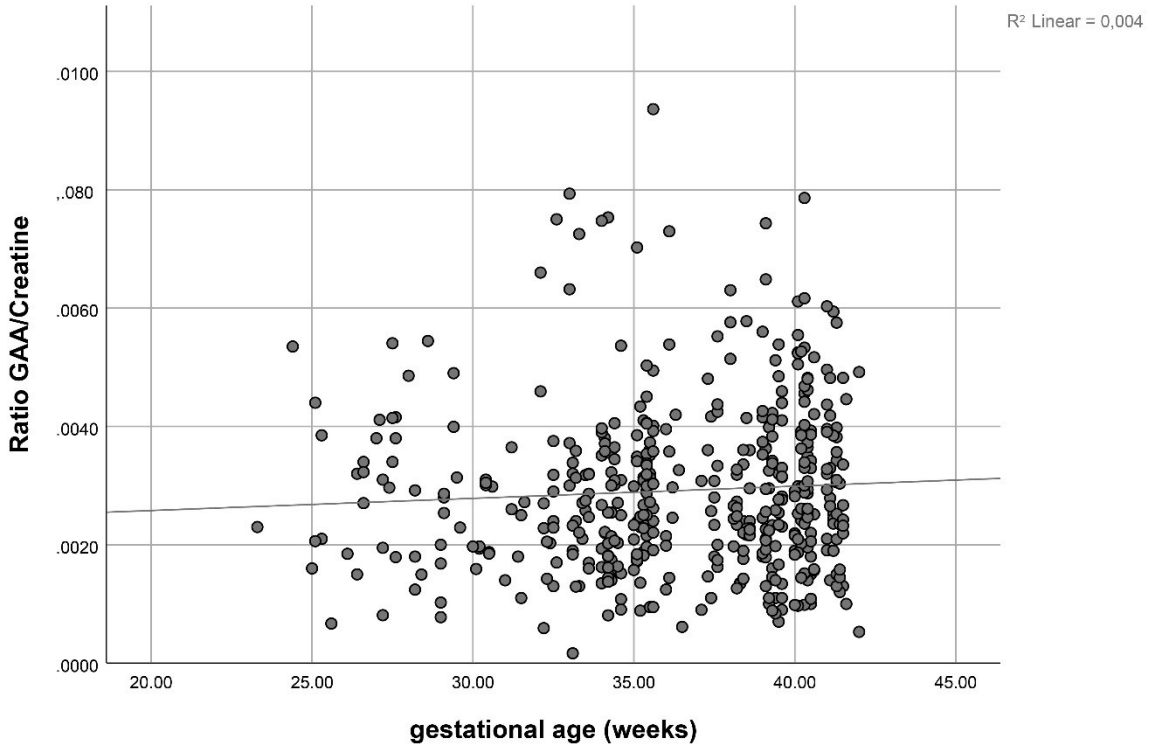
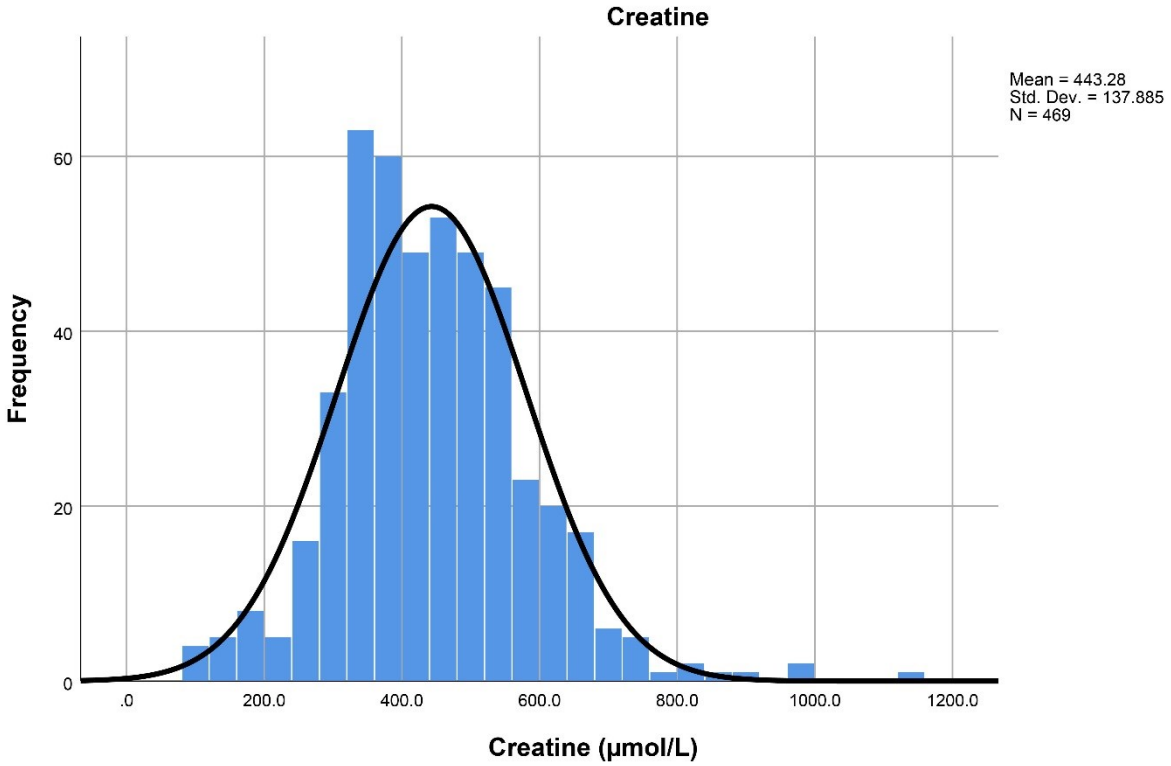
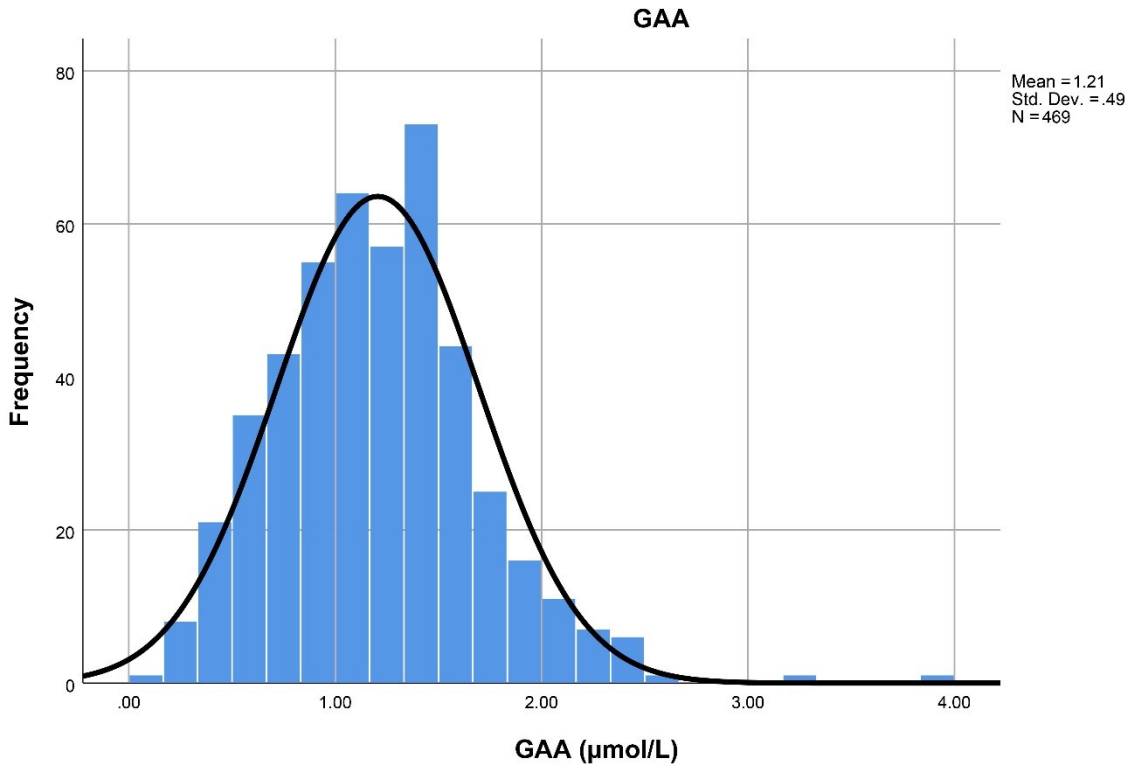


Figure 20 Scatterplots of creatine and GAA concentration and creatine/GAA ratio versus duration of pregnancy for 469 newborn screening bsp samples.

3.3 Distribution

Since no difference in creatine and GAA concentrations between pregnancy duration and gender were found all groups were combined and showed a normal distribution is observed for creatine, GAA and their ratio (Figure 21).


Creatine
Mean = 443.28
Std. Dev. = 137.885
N = 469
Frequency
0
20
40
60
.0
200.0
400.0
600.0
800.0
1000.0
1200.0
Creatine (µmol/L)


GAA
Mean = 1.21
Std. Dev. = .49
N = 469
Frequency
0
20
40
60
80
.00
1.00
2.00
3.00
4.00
GAA (µmol/L)

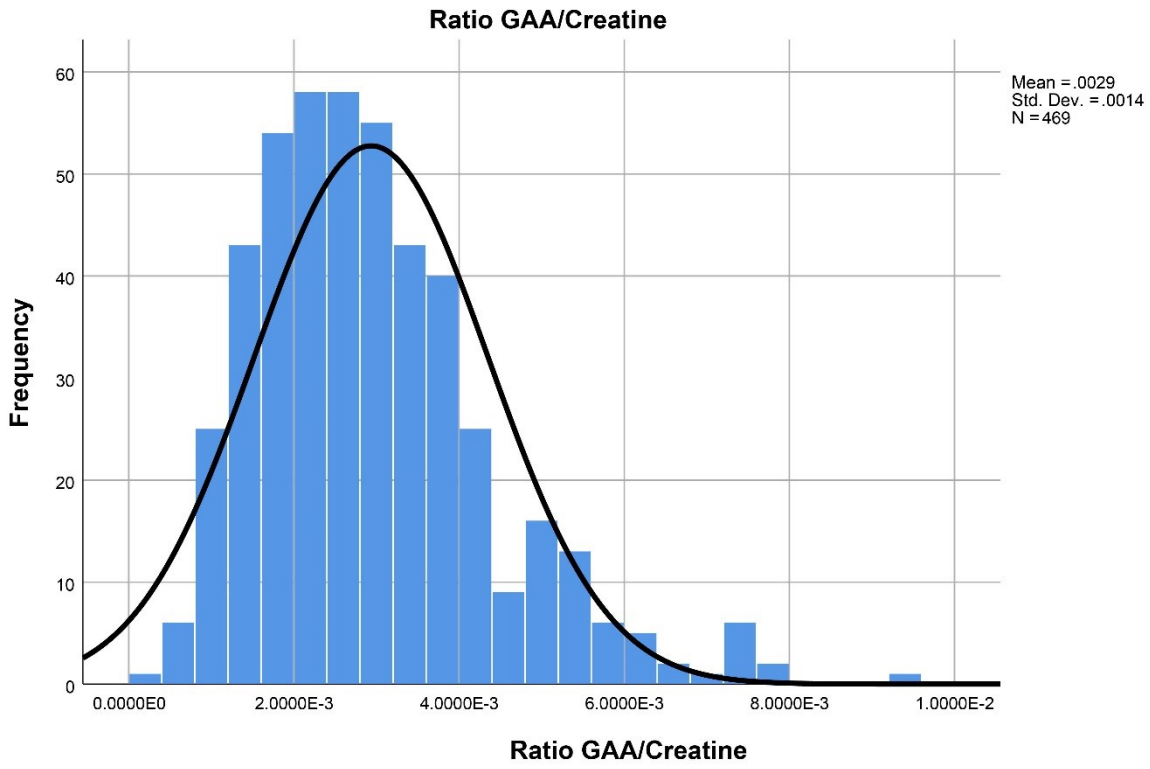


Figure 21 Histograms of creatine concentrations, GAA concentrations and the GAA/creatine ratio in newborn screening bsp samples (n=469).

The combined average of the 4 groups is listed in Table 19 and is in agreement with previous studies (Pasquali, Sinclair).

	Average	Std Dev
Creatine (uM)	443.3	137.9
GAA (uM)	1.21	0.49
GAA/Creatine	0.0029	0.0014

Table 20 Average concentrations of creatine and GAA and the GAA/creatine ratio of all groups combined (n=469).

3.4 Cut-off values

Since no difference was found between the different groups in the previous paragraphs, cut-off values were determined for all samples combined

Percentile	Creatine	GAA	GAA/Creatine	False positives
0.5	116.1			
1	125.4			
99		2.47	0.0075	4
99.5		3.06	0.0079	2

Table 21 Proposed cut-off values for GAA and creatine for GAMT-D detection in newborn screening BSP.

Both 99 and 99,5 percentile cut-off values resulted in lower GAA concentrations than previously reported (lowest 4.93 µmol/L, Pasquali 2014). For 99.5 percentile 2 sample were above the cut-off values (3.32 and 3.94 µmol/L). Addition of the GAA/creatine ratio did not lower the amount of false postive samples (same 2 detected for 99.5% for 99% ther were discrepancies between GAA and GAA/creatine false positives). A cut-off value for creatine should not be established because then inadvertly patients with AGAT deficiency could be found.

Conclusion
The 99,5 percentile cut-off value can be used for the detection of GAMT-D in the newborn screening. This concentration of 3.06 µmol/L falls in the range used in published method (range 2.94 – 5 µmol/L). The GAA/creatine ratio did not lower the number of false positive samples and thus has no added value in this method. Creatine could still be measured in this method and serve both as extraction efficiency control (low control GAA concentrations are below the LOQ) and a first indication that elevated GAA concentration are due to GAMT-D (low creatine concentration). Quantification of creatine seperately instead of the ratio is not allowed since inadvertly patients with AGAT deficiency could be found.

3.5 Positive control

An archived newborn bloodspot card of a biochemically and genetically confirmed GAMT-D patient (age 30 months?) was used to verify whether the new method would screen this bloodspot punch as positive. For creatine a value of 214.8 µmol/L was found and for GAA 13.64 µmol/L was found. The GAA/creatine ratio was 0,064. Both GAA and GAA/creatine are well above the cutoff values when using the 99 percentile cut-off. Creatine was is in the lower range of the controls as expected. This confirms that the method used is able to detect GAMT deficient neonates.

4 Conclusion

All parameters fulfilled the criteria and this in-house method detects GAMT-D patients adequately. A 99.5th percentile cut-off value for GAA is recommended and creatine, for now, does not seem to help lower the false positive rate.