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Lysosomal storage disorder 4+1 multiplex assay for newborn screening using tandem mass spectrometry: Application to a small-scale population study for five lysosomal storage disorders

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Abstract

Background—We sought to modify a previously published tandem mass spectrometry method of screening for 5 lysosomal storage disorders (LSDs) in order to make it better suited for high-throughput newborn screening.

Methods—Two 3-mm dried blood spot (DBS) punches were incubated, each with a different assay solution. The quadruplex solution was used for screening for Gaucher, Pompe, Krabbe and Fabry diseases, while a separate solution was used for Niemann–Pick A/B disease.

Results—The mean activities of acid-β-glucocerebrosidase (ABG), acid sphingomyelinase (ASM), acid glucosidase (GAA), galactocerebroside-β-galactosidase (GALC) and acid-galactosidase A (GLA) were measured on 5055 unidentified newborns. The mean activities (compared with their disease controls) were, 15.1 (0.35), 22.2 (1.34), 16.8 (0.51), 3.61 (0.23), and 20.7 (1.43) (μmol/L/h), respectively. The number of specimens that fell below our retest level cutoff of <20% daily mean activity (DMA) for each analyte is: ABG (6), ASM (0), GAA (5), GALC (17), and GLA (2).

Conclusions—This method provides a simplified and reliable assay for screening for five LSDs with clear distinction between activities from normal and disease samples. Advantages of this new method include significant decreases in processing time and the number of required assay solutions and overall decreased complexity.

Keywords

Newborn screening; Lysosomal storage disorder; Multiplex; Tandem mass spectrometry

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1. Introduction

Therapies for some lysosomal storage diseases (LSDs), including Pompe, Fabry and Gaucher diseases are available. Early presymptomatic detection and initiation of therapy reduce disease-related morbidity and mortality [1–3]. Infants with infantile Krabbe disease may benefit from a cord blood transplant, but only if transplanted within the first or second month of life [4]. Previously, Li et al. developed the use of electrospray ionization tandem mass spectrometry (ESI-MS/MS) with dried blood spots (DBS) for the multiplex analysis of a panel of lysosomal enzymes (acid-β-glucocerebrosidase, ABG; acid-galactosidase A, GLA; acid α-glucosidase, GAA; acid sphingomyelinase, ASM; galactocerebroside-β-galactosidase, GALC) that when deficient cause Gaucher, Fabry, Pompe, Niemann–Pick A/B and Krabbe diseases, respectively [5]. This method was refined by Zhang et al. to make it more suitable for newborn screening laboratories [6]. Variations of Zhang's method have been used to screen for each of these LSDs, except for Niemann–Pick A/B [7–10]. Scott et al. simplified the ESI-MS/MS method to yield a triplex assay of Pompe, Fabry and mucopolysaccharidosis-I (MPS 1) [11]. Scott's approach was to combine all substrate internal standard pairs into a single buffer to perform the enzyme functional test. This simplified Zhang's method, which required a single buffer for each enzyme reaction. More recently, Metz et al. simplified Zhang's assay by eliminating the liquid/liquid and solid phase extraction steps by utilizing online column clean-up capabilities of HPLC–MS/MS instrumentation [12]. Shortly thereafter they reported their results of neonatal screening for five lysosomal storage disorders [13]. In the US, Illinois, Missouri, New Jersey and New Mexico all have mandates to screen for Gaucher, Fabry, Pompe, Niemann–Pick A/B and Krabbe diseases. New York currently screens for Krabbe disease and we sought to develop a similar assay to Scott and coworkers ESI-MS/MS multiplexed assay. Our approach was to use our current GALC assay conditions as a foundation for the multiplex assay and to add sequentially as many of the other enzymes as possible while maintaining good separation between normal and diseased control specimens. Additionally, we wanted to minimize the number of buffers and DBS required for this assay. We then performed a small population study to determine enzyme activities for the associated enzymes.

2. Materials and methods

2.1. Samples and substrate

All experiments were conducted in compliance with Institutional Review Board guidelines. Vials of pre-mixed substrates (S) and internal standards (IS) for each enzyme and quality control DBS samples (low, medium and high) were provided by Dr. Hui Zhou (Centers for Disease Control and Prevention, Atlanta, Georgia). DBSs received for routine newborn screening purposes were used for the population study. Disease-positive DBSs were received from outside sources [Dr. X. Kate Zhang (Genzyme Corp.), Dr. C. Ron Scott (Univ. of Washington), Dr. David Kasper (Austria) and Dr. Joel Charrow (IL)], and consist of both newborn and adult samples from clinically diagnosed individuals. Due to limited availability of these positive controls, (only 1 punch provided) ASM controls were not tested for ABG, GAA, GALC or GLA, and one GLA control was not tested for ASM, as this would have required a second punch. All newborn DBS were shipped at room temperature and stored at 4 °C in plastic bags with desiccant until tested. Quality control DBS (LSD low, med and high) were provided by Dr. Hui Zhou (Centers for Disease Control and Prevention) and stored at −20 °C until tested [14]. Blank specimen cards were punched and assayed on each plate to perform blank subtraction. Two punches of each quality control DBS and 3 punches from blank specimen cards were included in each assay plate.

2.2. Preparation of assay cocktail solutions

We used two 3 mm DBS punches, one for the quadruplex assay (GALC, ABG, GAA and GLA) and one for the ASM assay. To prepare the quadruplex assay solution the contents of an ABG-S/IS, and a GAA-S/IS vial were dissolved in methanol (3 mL each) (JT Baker, NJ) and transferred to a GALC-S/IS vial, and the methanol was evaporated using in-house compressed air. The contents of a GLA-S/IS vial were dissolved in 1.8 mL of 96 g/L sodium taurocholate solution (Sigma cat. no. T4009, USA) and transferred to the ABG/GAA/GALC combination vial.

The vial was vortexed and heated in a hot tap water (~50 °C) bath until the contents completely dissolved. Acarbose (0.3 mL, 0.8 mmol/L, Toronto Research Chemicals, Ontario, Canada) and 0.2 mol/L sodium phosphate/0.10 mol/L citrate buffer (15.9 mL, pH 4.4, Sigma-Aldrich, St. Louis, MO), were added to the vial and the resulting solution was vortexed.

GLA inhibitor (1 M N-acetyl-D-galactosamine) was not included in the multiple analyte solution used in this study due to recent data showing it was unnecessary [11]. The ASM solution was prepared in the same manner as described previously using sodium acetate (Sigma-Aldrich), zinc chloride (Sigma), glacial acetic acid (Fluka, Seelze, Germany) and sodium hydroxide (Krackeler Scientific #11-3722-04, NY, USA) [6]. Final assay cocktail compositions are in Table 1. For complete list of reagents used, solution recipes and storage conditions see Supplementary data.

2.3. Sample processing

DBSs were punched into duplicate 96-well v-bottom microtiter plates (Costar V-Bottom #3363, Corning NY) using a Wallac DBS Puncher (Perkin Elmer, Waltham, MA). One plate received 30 μL of quadruplex assay cocktail using a 12-channel pipette (Biohit Proline 25–250 μL), while the other plate received 30 μL of ASM cocktail. Plates were sealed with a polypropylene plate sealer (Costar #3080, NY) and centrifuged at 2000 RPM (Eppendorf, Centrifuge 5810) for 2 min prior to being incubated at 37 °C for 19 h with shaking at ~160 RPM (Barnstead Titer Plate Shaker #4625, Dubuque, IA).

All post-incubation processing, including all pipetting steps and the solid phase extraction (SPE), was performed using specially programmed Biomek® NX Laboratory Workstations (Beckman Coulter, USA) and Cytomat hotels (Kendro, Langenselbold, Germany), which allowed for maximum processing efficiency.

Reactions were quenched with 100 μL of 1:1 methanol/ethyl acetate per well (Axygen, 250 μL pre-sterilized tips, LRS, Union City, CA); and 100 μL aliquots from duplicate wells on each plate were combined into a single well of a deep-well plate. Ethyl acetate (400 μL per well, JT Baker, Phillipsburg, NJ) was added followed by 400 μL of water (Barnstead Filtration System, Nanopure Diamond Filter), and the plates were centrifuged (2 min, 2000 RPM). The upper layer (150 μL) was transferred to a new 96-well plate, and solvent was evaporated at 35 °C using a TurboVap 96 (Caliper Life Science, Hopkington, MA). Samples were reconstituted in 150 μL of 19:1 ethyl acetate:methanol and transferred for solid phase extraction (SPE) to a 96-well filter plate (E & K Scientific Santa Clara, CA) pre-packed with 90–100 mg of silica (Sigma-Aldrich #227196, 230–400 mesh, 60A, Merck, Grade 9385) per well. A vacuum manifold designed for use with the liquid handling equipment was used to perform the SPE; the eluant was collected in a 96-well deep-well plate (Costar). Solvent was evaporated as above, and residues were reconstituted into 130 μL of 5 mmol/L ammonium formate in 80:20 methanol:water (Sigma-Aldrich) and transferred to a 96-well plate (Costar). The plate was wrapped with aluminum foil and placed into the auto-sampler for ESI-MS/MS analysis. For a complete list of the materials used, see Supplementary data.

2.4. LC–MS/MS enzyme analysis

The ESI-MS/MS was performed on a Quattro *micro*™ triple-quad mass spectrometer (Waters, Milford, MA) used in positive ion mode (see Supplementary data for specific instrument settings). Injections of 20 μL were done with a variable flow-rate using 80:20 methanol/water on a Waters 1525 μ binary HPLC coupled with a Waters 2777C sample manager. The following variable flow-rate was used: 0.1 mL/min for 0.05 min after injection, decreasing to 0.04 mL/min over 0.15 min, increasing to 0.08 mL/min over 0.55 min and then ramping up to 0.6 mL/min in 0.15 min for 0.1 min to completely flush the sample from the system before returning to 0.1 mL/min prior to the next sample injection. Data was collected during 1 min of infusion and signal returned to the background between injections. Data was acquired and analyzed by MassLynx™ 4.0 software.

2.5. Population study using Zhang method

Prior to creation of the 4+1 multiplex assay over 6700 anonymized specimens were tested (in 2008) using the previously published MS/MS method from Zhang and coworkers (with minor modifications to volumes transferred due to 200 μl pipette capacity; see Table 1 of Supplementary data for list of modifications) for ABG, GAA, GALC, and GLA. This study did not include the ASM assay.

3. Results

3.1. Enzyme activity

The mean enzyme activities and standard deviations for ABG, GAA, GALC, GLA and ASM measured in 5055 anonymized newborn DBSs along with values for disease-positive DBSs are summarized in Table 2. The blank corrected enzyme activity for each specimen was calculated in units of μmol/L/h, [(P/IS)*[IS]*(volume of IS μL/volume blood μL)*(1/incubation time h)] assuming that a 3.2-mm DBS contains 3.4 μL of blood. Activities were then converted into a percentage of the daily mean activity (% DMA) for each analyte. Specimens with <20% DMA were recorded but were not retested for confirmation because they were de-identified prior to analysis. Twenty percent of DMA is likely to be a conservative cutoff based on the current NYS Krabbe newborn screening algorithm. For Krabbe screening, specimens with an activity level<20% DMA are retested in duplicate punches from the original screening card. Specimens with an average activity ≤ 12% DMA are submitted for DNA analysis. These infants are referred for a diagnostic work-up if the average of activities is ≤ 12% and one or more mutations are detected [8]. The minimum activity detected among the anonymized normal DBSs exceeded the maximum activity among the disease-positive DBSs for all 5 enzymes by >50% (Table 2). All disease-positive DBSs had activities that were less than 11% DMA for the corresponding enzyme and most were <7% DMA. Of the 5055 normal DBSs tested, the number of specimens falling below the (20% and 12% DMA) cutoff for each analyte was ABG (6,1), ASM (0,0), GAA (5,1), GALC (17,2), GLA (2,0). Assuming screening of 250,000 specimens per year, and using the cut-offs established for Krabbe disease screening, the prospective yearly retest[1] and referral rates[2] (≤ 20%, ≤ 12%) for each analyte would be ABG (296, 49), ASM (0, 0), GAA (247, 49), GALC (839, 99), and GLA (99, 0). Actual disease cutoffs and referral rates would need to be established by performing a larger population study in the screening laboratory.

[1]Note that retests do not involve communication with parents or the medical community; these retests are conducted on the specimen already in-house.

[2]Also note that in NYS, incorporation of a second-tier molecular test for infants whose values are ≤12% DMA reduces referral rates by 60%; only those infants that carry one or more variants are referred to the medical community for diagnostic testing.

3.2. Quality control specimen data

Over 100 CDC LSD QC specimens were tested and data showed good distinction between high and low activity levels for all analytes. CDC LSD QC High specimens had the following analyte activities (standard deviations): ABG 7.46 (1.71), ASM 8.94 (1.51), GAA 12.2 (1.26), GALC 2.30 (0.25), and GLA 18.8 (1.73)μmol/L/h. CDC LSD "QC Low" specimens had the following analyte activities (standard deviations): ABG 0.92 (0.23), ASM 0.74 (0.38), GAA 1.13 (0.17), GALC 0.21 (0.09), and GLA 1.51 (0.19)μmol/L/h. The mean activity values for these QC specimens as determined by the CDC (using Zhang's method) are (High, Low): ABG (12.59, 0.99), ASM (2.54, 0.28), GAA (15.76, 1.13), GALC (3.47, 0.25) and GLA (8.31, 0.56) μmol/L/h (Supplemental Table 2) [14]. The 174 blank specimens had an average background activity of <0.15 μmol/L/h for all analytes. For a summary of all QC specimen results collected using the 4+1 method, see Supplementary data (Supplemental Tables 3A–3D).

3.3. Population study using Zhang method [6]

The mean activities of anonymized normal newborn DBSs measured using Zhang's method (compared to mean activities of the modified method) for ABG, GAA, GLA, and GALC, were 27.1 (14.1), 16.5 (16.8), 10.9 (20.7), and 3.61 (3.61), respectively (Table 3).

4. Discussion

4.1. Elimination of DBS extraction

We modified the method of Zhang and coworkers [6] by eliminating the DBS extraction step and significantly reducing the number of assay solutions (from 5 to 2). Since GALC has the lowest specific activity for this assay, we started with the optimal buffer for this enzyme and sequentially added the other S/IS pairs for the other enzymes. We found it necessary to assay ASM separately because the activity of this enzyme decreases substantially in the GALC buffer.

When comparing the results of our 4+1 method to those we obtained using the original method (Section 3.3), there are 2 main variables that could explain the difference in enzyme activities between the two methods. In this method, we used whole DBS instead of a pre-extracted DBS, and we used only a single buffer for the 4 enzymes instead of a separate buffer for each enzyme.

The 4+1 multiplex method eliminates the first 2 steps in Zhang's method [6] that includes a DBS extraction followed by distribution to separate assay mixtures. In doing so, we found that the measured enzyme activities in the DBS extracts were variable. With our automated liquid handling equipment, the order in which the DBS extracts are distributed to the plates affected the enzyme activities (Table 4, for experimental details see Supplementary data). If the order of distribution remained constant, we were able to get consistent results using the liquid handlers. This could result in data discrepancies between laboratories and will complicate values obtained when retests are conducted for selected analytes. By adjusting the processing solution to allow several enzymatic reactions to occur simultaneously on a single DBS, the need for DBS extraction has been eliminated. This modification effectively removed the impact of aliquot distribution order, which was likely due to the incomplete solubilization of enzyme, resulting in an inhomogeneous distribution of the enzyme in the extract reactions. The basis of this may result from co-localization of the enzyme with blood solids that are carried over when the extracts are distributed. To distribute the extract, the solution was mixed and distributed immediately to the ABG, GAA, than GLA plates (Table 4, Plate 3). This way the ABG reaction gets the most blood solids, and GLA gets the least amount of blood solids, which may explain why the average ABG activity was higher and

the average GLA activity was lower in this study compared to Zhang's method [6]. The average GAA activity was the same using both methods, as was GALC activity; the GALC assay is performed on a separate punch in both methods [6]. When the activity of the GLA and ABG controls is converted to %DMA as a surrogate (see Tables 2 and 3), enzyme results between the assays are comparable (e.g. for 2.33% vs 3.20% for ABG).

4.2. Optimization of solid-phase extraction

Attempts to eliminate the solid-phase extraction step were unsuccessful as the ABG substrate interfered with quantification of GALC and ABG products to internal standard ratios (data not shown). We were able to optimize the solid phase extraction step by significantly reducing the solvent volumes used for the solid-phase extraction step through silica when compared to the previously published assay [6]. This decreased the pre-ESI-MS/MS processing time by ~3-fold for the revised method. Despite these reduced solvent volumes, removal of buffer salts and detergent was sufficient such that no deterioration in ESI-MS/MS performance was observed, and very low background signals were obtained with blank DBSs. Finally, we observed that, when using the 4+1 multiplex assay, after running 5055 samples on one mass spectrometer over 1 week, that the electrospray ion source remained clean despite that fact that more analytes were being processed compared to our single assay for GALC. Metz et al. recently reported a simplified newborn screening protocol for lysosomal storage disorders that eliminates the need for the liquid/liquid and SPE clean-up steps by performing on line HPLC–MS/MS clean-up instead [12]. However, their protocol still required an enzyme extraction step, the extract had to be dispensed to 5 separate reaction tubes. Both of our approaches have incorporated improvements to streamline previous methods. Our approach requires more complicated sample clean-up; however, Metz's approach offers more complicated sample preparation (multiple buffers) and MS/MS methodology (HPLC separation). Which approach is used by others ultimately will likely be dependent on local capabilities and expertise.

5. Conclusions

Using this 4+1 multiplex assay, the total processing time, excluding the 19-h incubation, was reduced from 4 to 1 h. One technician was able to test over 5000 DBSs in 6 days, in addition to the usual workload, using equipment already existing in the laboratory showing that it is a highly efficient process. While automation of the liquid-handling steps for the assay makes it possible to routinely screen over 2000 newborns per day, the use of automated equipment is not required for this assay to be performed effectively. The modifications described here significantly decreased the total sample processing time and reduced the number of assay solutions, the amount of labware and solvent volume necessary for pre-ESI-MS/MS clean-up, while maintaining good distinction between normal and disease-positive DBSs. This high throughput method is feasible for screening for multiple LSDs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

LSD	lysosomal storage disorder
DBS	dried blood spot
DMA	daily mean activity
ESI-MS/MS	electrospray ionization-tandem mass spectrometry
ABG	acid β-glucocerebrosidase
ASM	acid sphingomyelinase
GAA	acid α-glucosidase
GALC	galactocerebrosidase
GLA	α-galactosidase
IS	internal standard
S	substrate
P	product

References

1. Schultz ML, Tecedor L, Chang M, Davidson BL. Clarifying lysosomal storage diseases. Trends Neurosci. 2011; 34:401–10. [PubMed: 21723623]
2. Hemsley KM, Hopwood JJ. Emerging therapies for neurodegenerative lysosomal storage disorders — from concept to reality. J Inherit Metab Dis. 2011; 34:1003–12. [PubMed: 21584766]
3. Wang RY, Bodamer OA, Watson MS, Wilcox WR. Lysosomal storage diseases: diagnostic confirmation and management of presymptomatic individuals. Genet Med. 2011; 13:457–84. [PubMed: 21502868]
4. Escolar ML, Poe MD, Provenzale JM, et al. Transplantation of umbilical cord blood in babies with infantile Krabbe's disease. N Engl J Med. 2005; 20:2069–81. [PubMed: 15901860]
5. Li Y, Scott CR, Chamoles NA. Direct multiplex assay of lysosomal enzymes in dried blood spots for newborn screening. Clin Chem. 2004; 50:1785–96. [PubMed: 15292070]
6. Zhang XK, Elbin CS, Chuang WL, et al. Multiplex enzyme assay screening of dried blood spots for lysosomal storage disorders by using tandem mass spectrometry. Clin Chem. 2008; 54:1725–8. [PubMed: 18719200]
7. Dajnoki A, Mühl A, Fekete G, et al. Newborn screening for Pompe disease by measuring acid alpha-glucosidase activity using tandem mass spectrometry. Clin Chem. 2008; 54:1624–9. [PubMed: 18703766]
8. Orsini JJ, Morrissey MA, Slavin LN, et al. Implementation of newborn screening for Krabbe disease: population study and cutoff determination. Clin Biochem. 2009; 42:877–84. [PubMed: 19318021]
9. Dajnoki A, Fekete G, Keutzer J, et al. Newborn screening for Fabry disease by measuring GLA activity using tandem mass spectrometry. Clin Chim Acta. 2010; 411:1428–31. [PubMed: 20338160]
10. Legnini E, Orsini JJ, Hung C, et al. Analysis of glucocerebrosidase activity in dry blood spots using tandem mass spectrometry. Clin Chim Acta. 2011; 412:343–6. [PubMed: 21070755]
11. Duffey TA, Bellamy G, Elliott S, et al. A tandem mass spectrometry triplex assay for the detection of Fabry, Pompe and mucopolysaccharidosis-I (Hurler). Clin Chem. 2010; 56:1854–61. [PubMed: 20940330]
12. Metz TF, Mechtler TP, Orsini JJ, et al. Simplified newborn screening protocol for lysosomal storage disorders. Clin Chem. 2011; 57:1286–94. [PubMed: 21771947]

13. Mechtler TP, Stary S, Metz TF, et al. Neonatal screening for lysosomal storage disorders. Feasibility and incidence from nationwide study in Austria. Lancet. 2012; 379:335–41. [PubMed: 22133539]

14. De Jesus VR, Zhang XK, Keutzer J, et al. Development and evaluation of quality control dried blood spot materials in newborn screening for lysosomal storage disorders. Clin Chem. 2009; 55:158–64. [PubMed: 18988750]

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2012.04.012.

Table 1

Final assay solution component concentrations using 4-plex+1 method

Quadruplex solution	ASM
6.67 μM GALC-IS, GAA-IS and GLA-IS, 13.33 μM ABG-IS	6.67 μM ASM-IS
1.0 mM GALC-S, 0.667 mM GAA-S, 3.33 mM GLA-S and 0.33 mM ABG-S	0.33 mM ASM-S
0.013 mM acarbose	0.8 g/L sodium taurocholate
9.6 g/L sodium taurocholate	0.83 M sodium acetate
0.18 M sodium phosphate and 0.09 M citrate	0.60 mM zinc chloride

Table 2

Enzyme activities in general population (n=5055) and disease-positive DBSs measured using the 4+1 multiplex ESI-MS/MS assay.

Analyte	Population mean (umol/L/h)	% DMA	Std deviation	Min	Max
GALC	3.6	–	2.23	0.43	25.8
GALC+ (n=2)	0.23	6.37	0.04	0.20	0.26
GAA	16.8	–	6.61	1.46	109
GAA+ (n=3)	0.51	3.02	0.30	0.21	0.81
GLA	20.7	–	9.71	3.48	106
GLA+ (n=3)	1.43	6.92	0.27	1.25	1.74
ABG	15.1	–	6.80	1.12	113
ABG+ (n=3)	0.35	2.33	0.18	0.17	0.53
ASM	22.2	–	8.57	5.31	125
ASM+ (n=3)	1.34	6.05	0.84	0.55	2.22

“+”indicates specimen was from an affected individual (n=number of affected individuals tested)

Table 3

Data collected for a population study performed using a modified, automated version of the previously published LSD multiplex method published by Zhang et al. [6]

Analyte	Population Mean (μmol/L/h)	% DMA	Std Deviation	Min	Max	N
GALC	3.61	–	2.72	0.21	60.64	6750
GALC+	0.35	9.73	0.11	0.21	0.61	11
GAA	16.53	–	7.39	0.03	143.77	6750
GAA+	0.30	1.83	0.23	0.01	0.62	8
GLA	10.94	–	6.85	0.42	145.47	6750
GLA+	0.51	4.62	–	0.51	0.51	1
ABG	27.14	–	16.38	0.43	656.54	6750
ABG+	0.87	3.20	1.88	0.43	1.57	3

Table 4

Shows how the order of extract distribution to analyte plates impacts enzyme activity levels.

Plate	Activity (μmol/L/h)			
	ABG	GAA	GLA	GALC
1	17.15	14.42	16.68	3.53
2	17.33	16.10	16.88	3.37
3	28.56	15.04	10.19	3.90

Plate # indicates the order of extraction distribution: Plates 1–2, GLA, GAA, then ABG and Plate 3, ABG, GAA, then GLA. GALC processing does not use an extract and therefore is not affected by the order of distribution