How Long are Residual Newborn Screening Specimens Useful for Retesting when Stored in Suboptimal and Uncontrolled Conditions of Temperature and Humidity?

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Abstract

Residual DBS specimens from newborns diagnosed with Phenylketonuria, Congenital Hypothyroidism, Cystic Fibrosis, Congenital Adrenal Hyperplasia and Galactosemia collected within 1995-2018, stored in cardboard boxes at ambient temperature in uncontrolled conditions, were retested for phenylalanine (Phe), thyrotropin (TSH), immunoreactive trypsinogen (IRT), total galactose (TGal) and 17-hydroxyprogesterone (17OHP), to demonstrate how long are they stable in these conditions and useful to reconfirm a previous abnormal result. Recovery percentage at retesting and qualitative interpretation regarding the current cutoff were evaluated. Phe, TSH and IRT recoveries showed decreasing trends along time. Phe recovery was 64 % after 2-years storage; TSH decayed rapidly recovering 47.3 % at 1-year, while IRT showed recoveries of 60 % at 1-year. Although 17OHP recovery presented a wide variation of results, a decaying trend was also found. Results suggest 17OHP is more stable than TSH and IRT, as supported by recoveries > 71 % when stored ≤ 2-years. TGal recovery presented an erratic behavior, so that it was not possible to estimate expected concentrations as a function of storage time. TGal recoveries above 100 % were found in UDP-galactose-4-epimerase and galactose-1-phosphate uridyltransferase deficiencies, evidencing possible galactose liberation from other sources. These results make a very valuable contribution for programs storing residual DBS in uncontrolled conditions.

Keywords: Newborn screening, Residual dried blood spots specimens, Analyte Stability, Storage conditions.

Introduction

Newborn screening (NBS) programs store residual dried blood spot (DBS) specimens for variable periods of time after routine testing has been completed. DBS specimens constitute a source of biological material characterized by its versatility, being usable in multiple applications that confer them a great potential as resource for public health programs [1].

The retention of residual DBS specimens in the short term has a primary justification whose objective is to document that a specimen was collected, received, and properly analyzed providing the possibility to verify the specimen quality and the number of blood discs punched [2]; and to make available a suitable material for retesting when NBS results must be validated in case of false negative results. Reanalysis of original DBS may be the only way to ascertain and document if a specimen mix-up or other events have taken place [1].

Over the years, secondary uses that justify their storage in the medium-term were added, such as quality assurance and retrospective diagnosis in cases of unforeseen morbidity and mortality during infancy; and also in the long-term, like the implementation of epidemiological and research studies of interest for the public health, the development of genomic population biobanks, and other kinds of testing such as forensic studies or searching for exposure to infectious, pharmacological, toxicological or environmental agents [1,2].

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The optimal storage conditions of residual DBS specimen for periods shorter than 2 years are in refrigerator (0-8 °C), in low gas-permeable zip-closure plastic bags with desiccants and humidity indicators cards to assure humidity below 30 %. When storage will extend for more than 2 years, residual DBS must be stored frozen (≤ -20 °C). In both temperature conditions, the inclusion of negative and positive controls along with the newborn specimens is recommended. Unquestionably, the fulfillment of the recommended storage conditions is an essential requirement to guarantee the specimen integrity [1–3].

In practice, the time and conditions of storage vary amongst programs and regions depending on the local regulations and the residual DBS specimen policies defined by each jurisdiction. Indeed, a clear example of this is the situation in the USA where by 2012 more than 45 % of NBS samples were stored for more than 21 years including a group of seven states which stored them indefinitely, while the remaining 55 % were retained for variable periods from 1 month to 5 years [4].

The main limitation for the fulfillment of the optimal storage conditions requirements is determined by the accessibility to economic resources to cover the associated costs and the availability of the appropriate refrigerated chambers. In developing countries like Argentina, the storage conditions usually are suboptimal, mainly due to economic limitations and most notably when programs screen for more than 50,000 or 100,000 newborns per year. This is the case of the NBS Laboratory of the Fundación Bioquímica Argentina which annually screens for more than 160,000 newborns and whose biobank was started more than 25 years ago, currently having stored more than 4.6 million residual DBS specimens.

Experiences reporting the stability of different analytes in DBS stored in different storage conditions have been published by numerous authors [3–23]. However, little or inconsistent information is available about for how long residual DBS specimens are useful for retesting when storage is made in suboptimal and uncontrolled conditions of temperature and humidity in the long-term.

Objective

The objective of this work was to present the retesting results of residual DBS specimens from newborns screened in the NBS Laboratory of the Fundación Bioquímica Argentina and diagnosed with Phenylketonuria (PKU), Congenital Hypothyroidism (CH), Cystic Fibrosis (CF), Congenital Adrenal Hyperplasia (CAH) and Galactosemia in the period 1995-2018, and stored in suboptimal and uncontrolled storage conditions of temperature and humidity[1], with the aim to demonstrate how long the specimens are stable in the study conditions and useful to reconfirm a previous abnormal NBS result.

[1] Throughout this paper the term uncontrolled storage conditions will be used to refer to suboptimal and uncontrolled storage conditions of temperature and humidity.

Material and Methods

Weather Conditions

With the aim to have a quantitative reference about how the local weather conditions could have affected the integrity of the residual DBS specimens during the storage in uncontrolled conditions of temperature and humidity, the registries published by the National Weather Service from Argentina corresponding to the period 1981-2010 in La Plata city [24] were reviewed.

Figure 1 reports the monthly average variation of the mean, maximum and minimum temperatures, and relative humidity in La Plata corresponding to the period 1981-2010. Although this period covers only 62 % of the complete storage period evaluated, information was extrapolated to the total period of study.

The annual temperature variations observed are in agreement with regional weather conditions, characterized by four seasons. Figure 1 shows that for 4 months per year (December – March), the average maximum temperature was above 25 °C.

Very high monthly averages for humidity were seen throughout all the year, with minimum average humidity of 72.9 % in warm months and maximum average of 83.4 % in cold months. In both cases average environmental humidity was higher than the optimal humidity conditions recommended for residual DBS specimens storage (< 30 %).

DBS Specimens Selection and Measurement Methods

Five hundred ninety nine residual DBS specimens collected along the period 1995-2018 from newborns diagnosed with PKU, CH, CF, CAH and Galactosemia, stored at room temperature in cardboard boxes, in suboptimal and uncontrolled conditions of temperature and humidity and without the addition of desiccants, were recovered and retested for phenylalanine (Phe), thyrotropin (TSH), immunoreactive trypsinogen (IRT), 17-hydroxyprogesterone (17OHP) and total galactose (TGal) depending on each newborn diagnosis.

Methods used for initial testing and retesting are described in Table 1. Retesting of all specimens for each analyte was made in January 2019 in singlicate, in a single analytical run to minimize the interassay variability.

As it is shown in Table 1, most of the residual DBS specimens were retested using the same method or at least the same analytical principle than those used at initial testing, this being an important fact in order to minimize the inherent variation due to the use of different methodologies. Exceptions to this statement were residual DBS specimens initially tested for Phe in 1995-1997 and for TGal in 1998-2000, given that the used measurement methods were based on different analytical principles than those used at retesting.

In the case of 17OHP, although initial testing and retesting were made using commercial reagents based on the same analytical principle, the method performance showed variations along the time due to changes in the kits versions design, which in some cases provided immunochemical reagents with poor specificity.

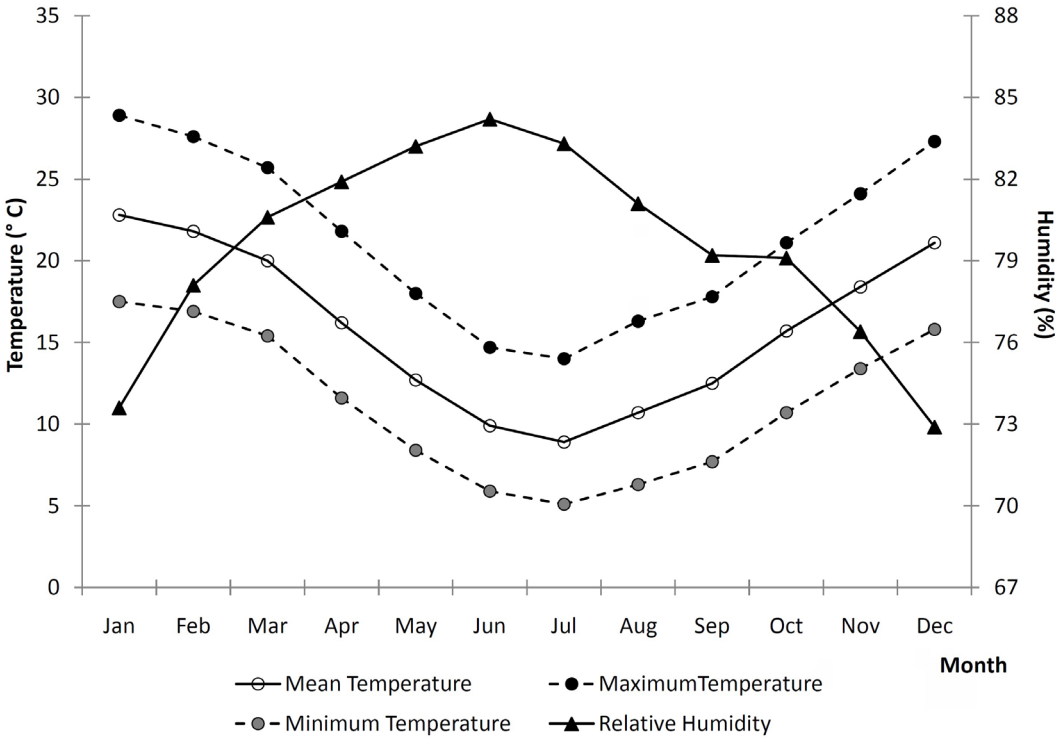

Figure 1. Monthly average variation of the mean, maximum and minimum temperature and relative humidity in La Plata corresponding to the period 1981-2010.

Table 1. Methods used for initial testing and retesting of DBS specimens included in the study.

Analyte	Period	Method		Cutoff at retesting
		Initial testing	Retesting	
Phe	Jan/95 – Apr/97	Bacterial inhibition assay (in-house)	Fluorometric (in-house)	2.5 mg/dL
	May/97 – Aug/98	Fluorometric. Labsystems Oy		
	Sep/98 – Jan/19	Fluorometric (in-house)		
TSH	Jan/95 – Jun/97	DELFIA. PerkinElmer	AutoDELFIA. PerkinElmer	11.0 µU/mL blood
	Jul/97 – Jan/19	AutoDELFIA. PerkinElmer		
IRT	Jul/95 – Jul/00	DELFIA. PerkinElmer	AutoDELFIA. PerkinElmer	70.0 ng/mL blood
	Aug/00 – Jan/19	AutoDELFIA. PerkinElmer		
17OHP	Dec/97 – Mar/00	DELFIA. PerkinElmer	AutoDELFIA. PerkinElmer	22.3 nmol/L blood
	Apr/00 – Jan/19	AutoDELFIA. PerkinElmer		
TGal	Jul/98 – Apr/00	Enzymatic Colorimetric. ICN Biomedicals Inc	Enzymatic fluorometric (in- house)	8.0 mg/dL
	May/00 – Jan/19	Enzymatic fluorometric (in- house)		

Note: Analyte cutoffs for the methods used at retesting are given for each biochemical marker.

Table 2 illustrates the distribution of residual DBS specimens stored in suboptimal and uncontrolled conditions of temperature and humidity selected for retesting for each group of disorders. Information about the range and mean of the analytes concentrations at initial testing is also included.

Additionally, a complementary set of 142 residual DBS specimens from newborns diagnosed with the same five disorders, collected in the period 1996-2006 and stored at -20 °C in aluminized-plastic bags with desiccants, were recovered and tested in singlicate in the same run, in order to establish a comparison amongst both storage conditions (Table 3). These specimens were stored at -20 °C immediately after the initial testing for another purpose, so that the storage time ranges were different for each disorder.

Table 2. Distribution of selected residual DBS specimens stored in uncontrolled conditions, according to the diagnosis.

Disorder	N° of DBS specimens	Analyte (units)	Range (Mean)
Phenylketonuria	117	Phe (mg/dL)	6.0 – 19.5 (9.4)
Congenital Hypothyroidism	116	TSH (µU/mL blood)	20.0 – 413.6 (132.8)
Cystic Fibrosis	68	IRT (ng/mL blood)	145.5 – 249.2 (194.3)
Congenital Adrenal Hyperplasia	140	17OHP (nmol/L blood)	22.9 – 995.0 (277.5)
Galactosemia	Partial GALT deficiency: 121	TGal (mg/dL)	8.0 – 50.4 (14.5)
	Severe GALT deficiency: 17		13.6 – 100.0 (66.8)
	GALK deficiency: 3		25.6 – 80.0 (46.6)
	GALE deficiency: 16		9.3 – 36.2 (15.9)

Note: Details about range and mean concentrations are included for each analyte.
GALT: Galactose-1-phosphate uridyltransferase, GALK: Galactokinase, GALE: UDP-Galactose 4-epimerase.

Table 3. Distribution of selected residual DBS specimens stored in freezer at -20 °C, according to the diagnosis.

Disorder	N° of DBS specimens	Analyte (units)	Range (Mean)
Phenylketonuria	33	Phe (mg/dL)	6.0 – 19.8 (9.1)
Congenital Hypothyroidism	30	TSH (µU/mL blood)	21.2 – 357.0 (131.5)
Cystic Fibrosis	19	IRT (ng/mL blood)	153.9 – 249.6 (198.9)
Congenital Adrenal Hyperplasia	11	17OHP (nmol/L blood)	76.0 – 766.0 (328.2)
Galactosemia	Partial GALT deficiency: 43	TGal (mg/dL)	8.3 – 44.3 (14.8)
	Severe GALT deficiency: 4		50.0 – 150.0 (104.5)
	GALK deficiency: 2		21.6 – 170.0 (95.8)

Note: Details about range and mean concentration are included for each analyte.
GALT: Galactose-1-phosphate uridyltransferase, GALK: Galactokinase, GALE: UDP-Galactose 4-epimerase.

Results Evaluation

Results from retesting residual DBS specimens stored in suboptimal and uncontrolled conditions were compared with those obtained at initial testing. Recovery percentage at retesting was calculated and the current cutoff value was used to interpret results. Recovery percentage was plotted versus the storage time, and the current retesting results interpretation was matched with the initial one in order to determine whether retesting was still able to detect specimens as abnormal or not.

A similar evaluation was made for residual DBS specimens stored at -20 °C.

Results

Phenylalanine

All specimens included in the study were intentionally selected in the referral range in which most of the confirmed PKU forms cases are detected, being the reason why Phe levels at initial screening of selected specimens were ≥ 6.0 mg/dL

Figure 2 shows a clear linear decreasing trend of Phe recovery as storage time increased (r = 0.810). Maximum recovery was approximately 70-80 % in specimens stored for 2-3 years, and a reduction at approximately the half of the initial Phe concentration was observed in samples stored for 5-9 years.

The qualitative interpretation of Phe concentrations at retesting compared to the current cutoff value (2.5 mg/dL) showed that all samples initially tested before 2003 (> 16 years old of storage) had normalized their Phe levels at retesting (n = 20) (black circles on the right of the vertical dashed line in Figure 2). In contrast, 90 % of samples stored from 2003 onwards (88/97) yielded results above the current cutoff, and were interpreted as abnormal. Detailed analysis of initial Phe concentrations in 9/97 specimens in which Phe levels normalized at retesting (black circles on the left of the vertical dashed line in Figure 2) showed they all had Phe levels in the lowest selected range (6.0 to 7.1 mg/dL), highlighting a direct relationship between results normalization and initial Phe concentration. All specimens stored for ≤ 6 years (n = 33) presented abnormal results at retesting, irrespective of the Phe concentration at initial testing.

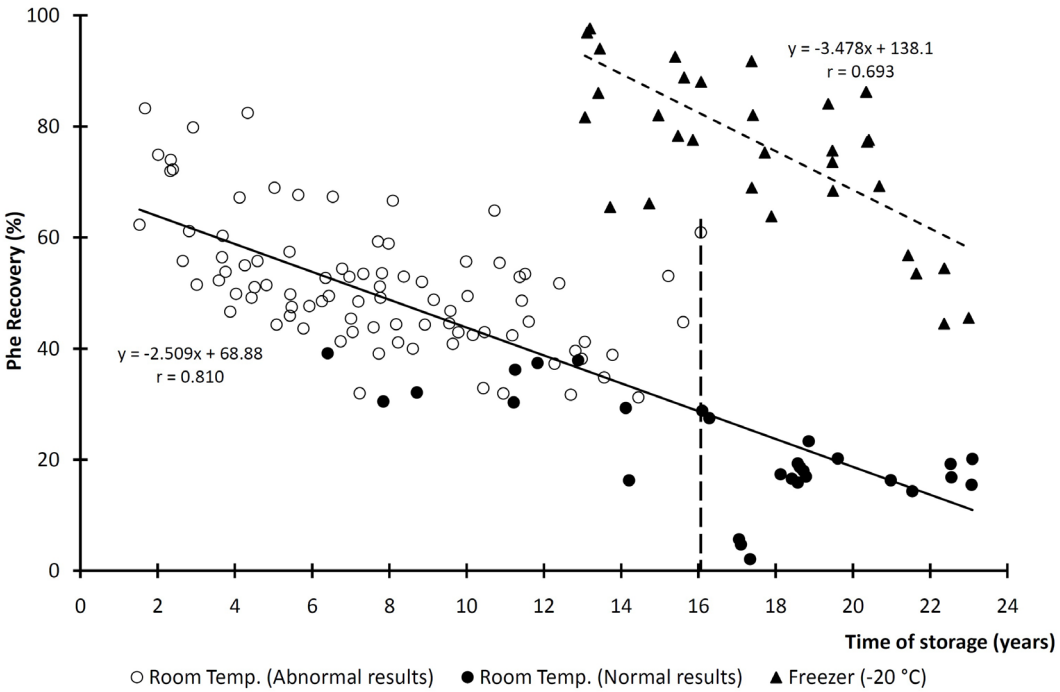

Figure 2. Phe recovery at retesting in residual DBS specimens stored in two different storage conditions. Note: the legend Abnormal/Normal results indicate the qualitative results interpretation at retesting.

Retesting results of 33 residual DBS specimen stored at -20 °C during the period 1996-2006 showed a decay curve similar to specimens stored in uncontrolled conditions but starting from a higher recovery percentage (dashed trend line on Figure 2). In fact, most of specimens stored at -20 °C for 13-18 years presented recoveries in the range 80-100 %, and 100 % of such specimens (33/33) presented Phe levels above the cutoff value, thus being interpreted again as abnormal. The recovery decayed to approximately 40-50 % only in DBS stored for 21-24 years.

TSH

Residual DBS specimens from newborns affected with CH having a wide TSH analytical range were selected for retesting and grouped into three ranges: low [(20.0 – 35.3 µU/mL), mean = 28.1 µU/mL, n = 38], medium [(55.7 – 75.0 µU/mL), mean = 66.6 µU/mL, n = 39], and high [(229.2 – 413.6 µU/mL), mean = 301.3 µU/mL, n = 39]. For each one of these ranges 2 specimens per year were selected, except for the period 2001-2005 because all the specimens with elevated TSH detected in such period corresponding to CH confirmed cases were stored at -20 °C for another purpose immediately after the initial testing was completed.

Figure 3 shows that TSH recovery decayed rapidly during the storage, demonstrating that the TSH stability in residual DBS specimens is critically affected by uncontrolled storage conditions, and was independent of the initial TSH concentration. The experimental decay observed in TSH levels was adjusted with a logarithmic function (r = 0.906) which shows that recovery drops to 50-60 % in just 6-12 months of storage.

Furthermore, retesting results interpretation using the current TSH cutoff value (11.0 µU/mL) was strongly dependent on the initial TSH value. Specimens in low, medium and high TSH ranges showed abnormal results at retesting only when the time of storage was < 1 year, < 3 years and < 12 years, respectively. Moreover, 100 % of the residual DBS specimens with TSH in the low range (20.0 – 35.3 µU/mL) turned out normal when they were stored for ≥ 2 years.

With regard to the 30 residual DBS specimens stored at -20 °C during the period 2001-2005, it was observed that TSH recovery at retesting ranged from 40 to 95 % but distributed in a random way irrespective of the storage time and the TSH level at initial testing, not being possible to establish a decay pattern.

The qualitative interpretation of TSH results at retesting regarding the current cutoff value showed that 100 % of specimens stored at -20 °C had elevated levels of TSH and they were all identified as abnormal.

IRT

Figure 4 presents the decay curve for IRT recovery at retesting of residual DBS specimens during storage, adjusted using an order-5 polynomial function (r = 0.906).

Paradoxically, and despite the knowledge about the reduced stability of IRT at room temperature that in practice determine the loss of the diagnostic screening value of normal results when they are obtained from samples with more than 15 days from the collection [14,23], the IRT decay rate during the first 5 years of storage was less critical than the expected, showing

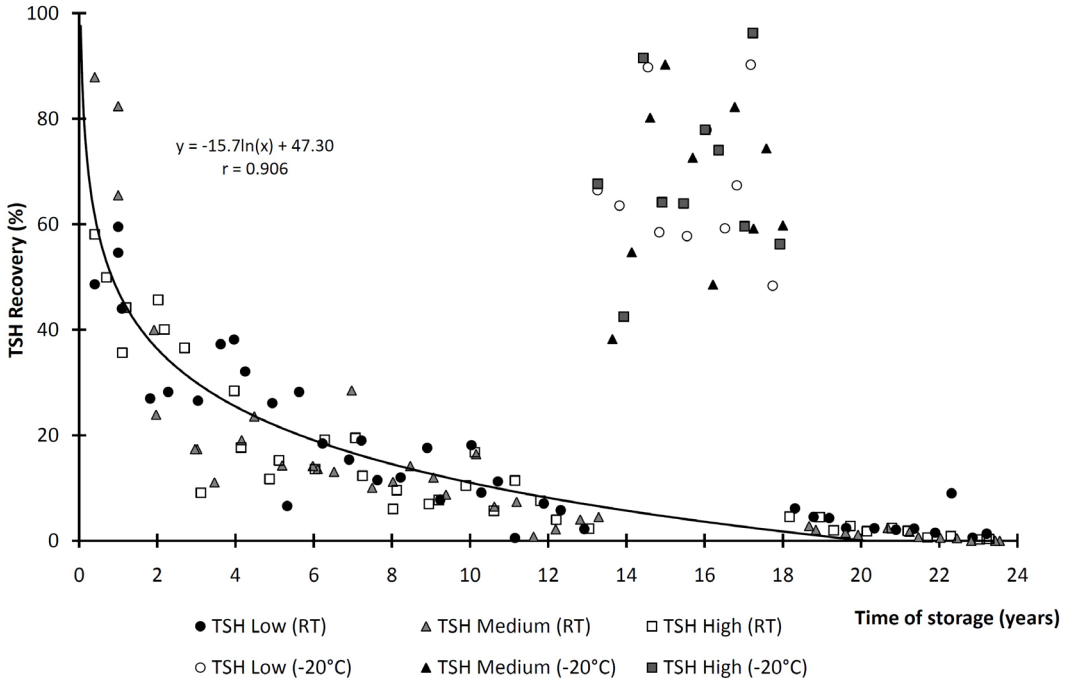

Figure 3. TSH recovery at retesting in residual DBS specimens stored in two different storage conditions. RT: room temperature.

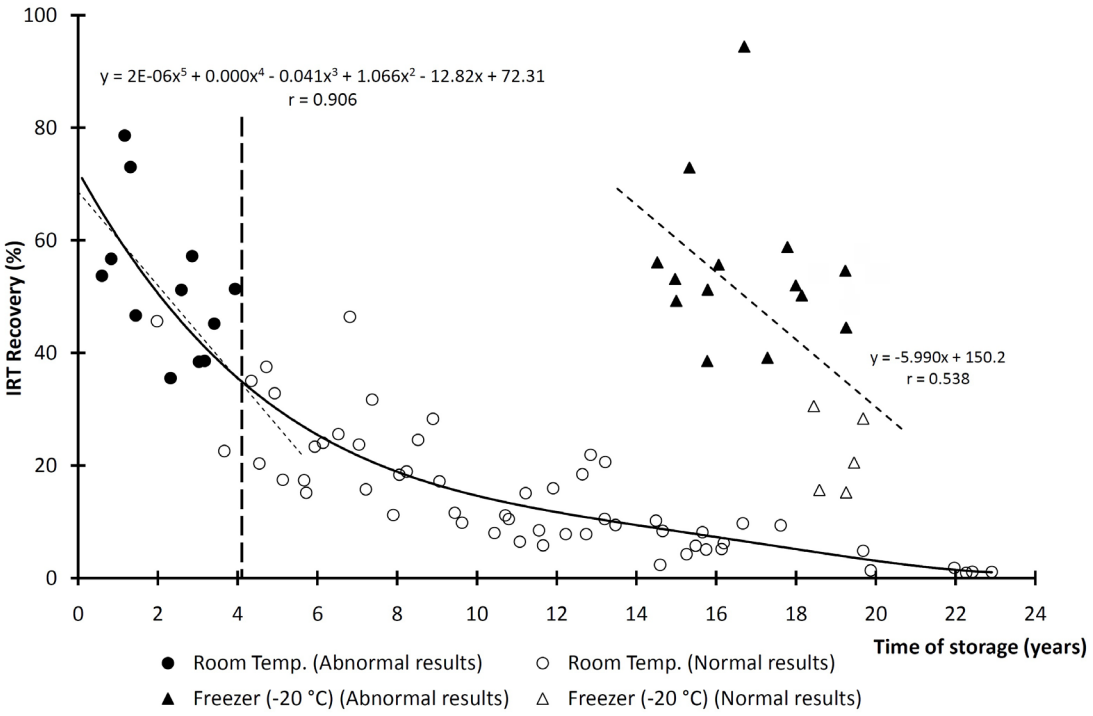

Figure 4. IRT recovery at retesting in residual DBS specimens stored in two different storage conditions. Note: the legend Abnormal/Normal results indicate the qualitative results interpretation at retesting.

a reduction in IRT recovery of approximately 50 % in samples stored from 1 to 4 years.

Results interpretation at retesting using the current cutoff value (70.0 ng/ml) showed that 79 % of residual DBS specimens analyzed (54/68) normalized their IRT concentrations. However, 86 % of specimens stored for 4 years or less (12/14) presented abnormal results at retesting (black circles on the left of the vertical dashed line on Figure 4), from which 3 had IRT levels in the lowest selected range (145.5 to 157.3 ng/ml).

Retesting of 19 residual DBS specimens stored at -20 °C during the period 1999-2004 showed a slight linear trend of decreasing in the IRT recovery. Even though the number of specimens in this group was small, both the slope of the linear trend curve and the points distribution at this storage condition for 14-20 years followed a similar decay pattern and points distribution to those observed during the first 5 years of storage in uncontrolled conditions.

As shown in Figure 4, 5/19 results (26.3 %) were normal when compared to the current cutoff value. Two of them had initial IRT values in the lowest selected range (158.4 and 163.6 ng/mL) and 2 were near the highest range (224.7 and 228.7 ng/mL), suggesting that the IRT normalization for specimens stored at -20 °C could be independent of the initial IRT concentration at the studied ranges.

17OH Progesterone

Given the variability of the 17OHP concentrations in the stored residual DBS specimens selected for retesting, they were divided into four ranges: low [(22.9 – 89.0 nmol/L), mean = 52.8 nmol/L, n = 39], medium [(91.3 – 223.4 nmol/L), mean = 151.8 nmol/L, n = 34)], moderately high [(241.0 – 443.4 nmol/L), mean = 332.7 nmol/L, n = 34], and high [(451.0 – 995.0 nmol/L), mean = 615.9 nmol/L, n = 33].

Unlike the behavior observed in the case of Phe, TSH and IRT for which the decay in the analyte recovery at retesting showed a pattern of points distribution relatively homogeneous around the trend curve, in the case of 17OHP the recovery at retesting in residual DBS specimen stored in uncontrolled conditions presented a wide dispersion, including 5 specimens for which recoveries were > 100 % (Figure 5). In spite of this, a linear trend of 17OHP recovery was drawn which as expected, presented a lower correlation coefficient (r = 0.582) than those corresponding to the three previously mentioned analytes.

When the graphic analysis of recovery as a function of the time of storage was made, it was possible to determine that specimens with initial 17OHP concentrations in the low, medium and moderately high ranges did not show differences in the points distribution each other and, therefore, they were plotted as a unique class (22.9 – 443.4 nmol/L) in Figure 5. In contrast, the behavior of specimens with initial 17OHP concentrations in the high range (451.0 – 995.0 nmol/L) was clearly different as marked by the dashed-line oval in Figure 5, inside of which fall 19/33 points corresponding to this range (black triangles), and also by the fact that only 9/33 results fall below the linear trend curve.

In semi-quantitative terms, DBS specimens stored for < 2 years (n = 11) presented recoveries higher than 71 %, while when stored for 2-5 years mostly were in the range 53-78 %.

To interpret results at retesting, the current cutoff value corresponding to newborns with birth weight ≥ 3650 g (22.3 nmol/L) was used. In this way, 84 % of the total residual DBS specimens retested (118/140), 99 % of those with initial 17OHP ≥ 74.5 nmol/L (109/110) and 100 % of the specimens with initial 17OHP > 125 nmol/L (90/90), were again classified as abnormal, irrespective of the time of storage (1 month to 21 years).

As can be seen in Figure 5, residual DBS specimens whose 17OHP at retesting were lower than the cutoff value (black circles, n = 22) were found scattered along the storage range, behavior strongly dependent on the 17OHP concentrations at initial testing. Twenty of 22 DBS specimens that belonged to this group had 17OHP at initial testing in the low range (22.9 – 55.2 nmol/L). Only 1 specimen with 17OHP higher than 100 nmol/L (125 nmol/L) had a normal result at retesting. This finding was probably due to the use of the AutoDELFIA kit B015-112 at initial testing, version characterized by its documented poor specificity that could be linked to the high initial 17OHP value.

The retesting of the 11 residual DBS specimens stored at -20 °C during the period 1999-2003 showed a random distribution in 17OHP recovery (Figure 5), they all being interpreted as abnormal when compared with the current cutoff value.

Total Galactose

As it was illustrated in Table 1, the residual DBS specimens selected for retesting were grouped according to the deficient enzyme causing the disorder because each one of these deficiencies is characterized by a different pattern of abnormal analytes. The number of specimens in each group was directly related to the incidence of each enzyme deficiency. In the case of the galactose-1-phosphate uridyltransferase (GALT) deficiency, the group was divided according to the severity of the deficiency in partial and severe (classic galactosemia).

Figure 6 shows random percentage recoveries of TGal at retesting of residual DBS specimens stored in uncontrolled conditions, behavior difficult to explain in technical or analytical terms. In fact, 35 % of retested specimens (55/157) showed recoveries ≥ 100 %, and 64 % (101/157) ≥ 85 %, irrespective of the storage time. Such behavior does not allow any kind of adjustment or results systematization that helps to estimate the expected TGal concentration as a function of the time of storage in uncontrolled conditions.

Nevertheless, there are two constant findings to highlight. First, 3 DBS specimens corresponding to newborns affected by a Galactokinase (GALK) deficiency presented recoveries below 12.5 % (black squares on Figure 6), and second, the recovery in the 16 DBS specimens corresponding to newborns affected by an UDP-galactose 4-epimerase (GALE) deficiency was, on average, 138 % and only 3 of these 16 specimens presented recoveries below 100 % (range 76 - 96 %).

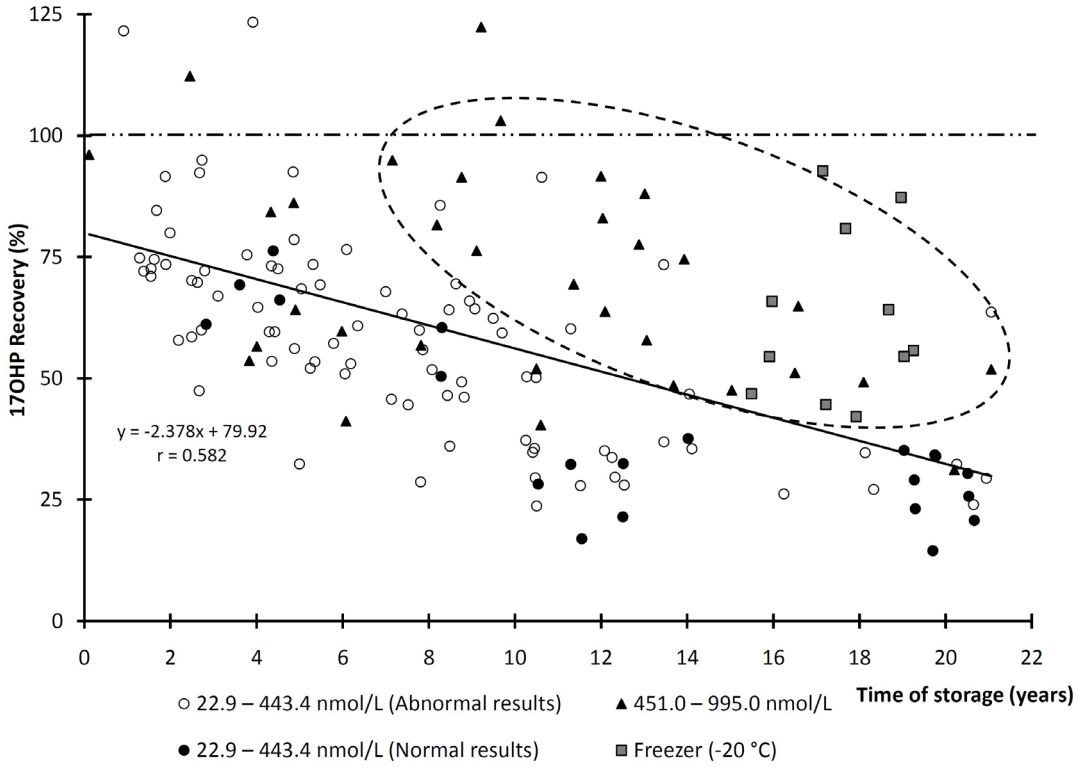

Figure 5. 17OHP recovery at retesting in residual DBS specimens stored in two different storage conditions. Note: References ○, ● and ▲ correspond to residual DBS specimens stored at room temperature. The legend Abnormal/Normal results indicate the qualitative results interpretation at retesting.

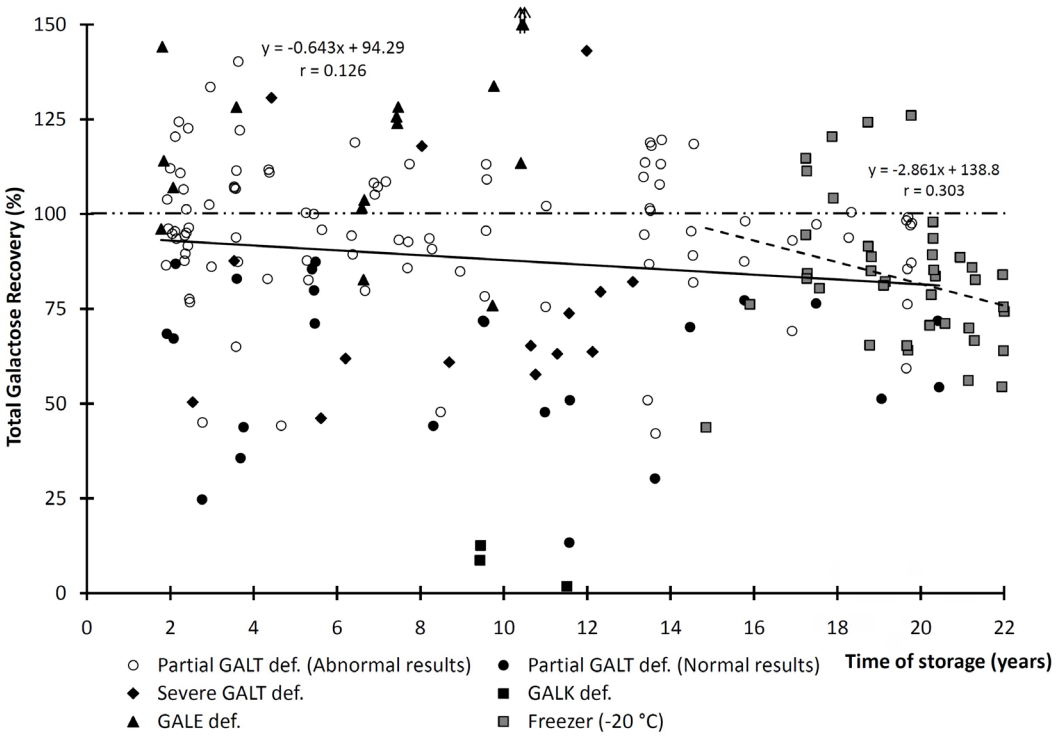

Figure 6. TGal recovery at retesting in residual DBS specimens stored in two different storage conditions. Note: References ○, ●, ♦, ■ and ▲ correspond to residual DBS specimens stored at room temperature. The legend Abnormal/Normal results indicate the qualitative results interpretation at retesting. GALT: Galactose-1-phosphate uridyltransferase, GALK: Galactokinase, GALE: UDP-Galactose 4-epimerase.

When TGal concentrations at retesting were compared to the current cutoff value (8.0 mg/dl), 100 % of the residual DBS specimens with severe GALT deficiencies and GALE deficiencies and stored in uncontrolled conditions gave abnormal results, while all the specimens corresponding to GALK deficiencies normalized their values. The retesting of specimens with partial GALT deficiencies, showed that 38 % of them (60/157) normalized their results irrespective of the storage time.

Finally, 49 residual DBS specimens stored at -20 °C during 1996-2006 showed a more logical recovery behavior than those observed for specimens stored in uncontrolled conditions as illustrated in Figure 6. Seven of 49 specimens showed recoveries > 100 % (104 – 126 %) upon retesting, presenting a slight decreasing linear trend as the time of storage was increasing (dashed linear trend), with recoveries above 50 % after 20 years of storage. One important finding to highlight in contrast with the storage at ambient temperature is that specimens from newborns affected by GALK deficiencies showed recoveries of 64.1 and 65.3 % thus demonstrating the integrity sample protection for this deficiency when DBS are stored at -20 °C.

The qualitative results interpretation at retesting of these 49 specimens showed that all samples corresponding to severe GALT (4/4) and GALK deficiencies (2/2) had abnormal results at retesting, while 21 % of those specimens corresponding to partial GALT deficiencies (9/43) normalized their results demonstrating a direct relationship with the storage time (data not detailed in Figure 6).

Discussion

Several studies have concluded suboptimal and uncontrolled temperature and/or humidity storage conditions have a detrimental effect on the stability of NBS analytes. Length and conditions of storage, methods used for measurements and type of specimens used for evaluation can make it difficult to compare published experiences.

Adam *et al.* (5) reported an experimentally controlled study on the stability of 34 NBS markers at one temperature condition (37 °C) and two of humidity (< 30 % and > 90 %) to create an environment of accelerated degradation. Their results showed that after one month of storage at 37 °C at high humidity, TSH was critically affected losing 63.7 % of its concentration, while IRT, 17OHP and TGal were affected in a lesser degree, with losses of 53.3, 41.9 and 39.6 %, respectively. Furthermore, these 4 analytes also experienced a significant degradation at low humidity, in the range 16.8 - 21.8 %. TSH degradation at high humidity was more than three times greater than its degradation at low humidity. In turn, Phe seemed to be more stable with losses of 33.5 and 6.9 % at high and low humidity storage conditions, respectively.

Murphy *et al.* [6] retested 4 groups of 78 screen-negative newborn DBS samples each, stored in controlled temperature conditions (21 °C), during different periods of storage for each group of 2-, 4-, 6-, and 12-months, respectively. Retesting was made for 49 analytes included in the routine NBS panel. The results showed that concentrations of 17OHP, TSH and Phe were consistent at 2- and 4-months when compared to baseline, but after 12-months only 17OHP was stable, while TSH and Phe experienced slightly decreasing concentrations.

Strnadová *et al.* [7] evaluated the long-term stabilities of amino acids and acylcarnitines along a period of 15-years in 660 DBS specimens from normal newborns, which were stored at room temperature in a dry environment in sealed and bagged envelopes. They found an exponential decrease of Phe and estimated a constant 5.7 % reduction per year.

Prentice *et al.* [8] evaluated the stability of 18 amino acids and acylcarnitines in DBS stored at -80 °C, -20 °C and room temperature (21 °C) in storage bags with no addition of desiccant for a 2-year period. They observed that Phe was stable in both freezer conditions, but lost 40 % of the basal concentration at room temperature after 2 years of storage.

Han *et al.* [9] investigated the stabilities of 21 amino acids in DBS stored under environmental conditions simulating a global health workflow along a short-term 30-day period at -20 °C, 4 °C, 25 °C and 40 °C under laboratory humidity (38 %), and at 25 °C and 40 °C at high humidity (75 %). They found that Phe showed larger time course concentration decreases at 40 °C than at 25 °C, and concentration decreases were greater at higher humidity at both, 25 °C and 40 °C, than under laboratory humidity, reaching reductions of about 10-15 and 20 % after 1 month of storage, respectively.

Chace *et al.* (10) investigated the stability of 5 amino acids in DBS reference materials stored in zip-closure plastic bags with desiccant packets and humidity indicator cards in the dark at -20 °C, 4 °C, ambient temperature and 37 °C. They found that the initial Phe concentration did not show significant loss of concentrations in the three first conditions, and a decrease of 15-17 % after 30 days of storage at 37 °C.

Waite *et al.* [11] evaluated the storage effect on two TSH, T_3 and T_4 controls stored during a period of 36 days in six different conditions: -20 °C, 4 °C, 25 °C and 37 °C with desiccants, room temperature (25 °C) at high-humidity, and ambient temperature (4 to 30 °C). They conclude that TSH in DBS was relatively stable for as long as 1 month under their most adverse conditions evaluated.

Coombes *et al.* (12) reported that TSH was stable in 21 DBS from adults for at least 30 days when stored at -20 °C, 4 °C and room temperature (19-26 °C), but lost 16 and 30 % of its concentration when exposed to 37 °C for 6-8 days and 26-30 days, respectively.

Magalhães *et al.* [13] analyzed DBS from 29 adults with different TSH levels, stored at -20 °C, 4 °C and 22 °C along a 1-year period of storage wrapped in aluminum foil envelopes. TSH values remained stable up to 30 days when stored at 22 °C and was stable up to 60 days when stored in refrigerator or freezer. After 1-year of storage the median reduction of TSH concentrations when DBS were stored at -20 °C, 4 °C and room temperature was 10.1, 16.8 and 47.7%, respectively. They conclude that if confirmation of initial results should become necessary after 6

months of storage, it is necessary to consider the possibility of reduced TSH concentrations due to loss of hormone stability, mainly in samples close to cutoffs levels.

Therrel *et al.* [14] reported the evaluation of the IRT stability at two different concentrations and under five different environments conditions: -20 °C, 4 °C, and 22 °C; and 27 °C and 35 °C at 80 % of humidity. Their results showed a sharp decrease in IRT concentration along the first week of storage at high humidity and 27 and 35 °C, reaching recoveries around 50-60 and 40-50 % after 1 month, respectively. While IRT seemed to be stable at -20 and 4 °C, the storage at 22 °C showed a decrease of 10-15 % after 1 month.

Li *et al.* [15] evaluated the IRT stability in one DBS control material stored at -20 °C, 4 °C, 25 °C and 37 °C with desiccants. They reported little-to-no IRT degradation at -20 °C and 4 °C for 1 year, while at room temperature IRT was stable by 1 month and lost 25 % of its concentration after 1 year of storage. In contrast, at 37 °C DBS lost about 10 and 50 % of IRT after 1 month and 1 year of storage, respectively.

Török *et al.* [16] retested 17OHP in 520 normal newborn DBS specimens stored for a period of 12-years under typical room conditions in a moderate climate, and they found small but significant decrease in the 17OHP concentration (3.2 % per year), concluding that 17OHP in DBS stored for more than a decade can reliably be used for retrospective examinations and population studies.

Grecsó *et al.* [17] analyzed 17OHP and other four steroids in DBS samples stored at -70 °C, -20 °C, 4 °C and room temperature for a 1-year period. Based on the criterion that one analyte was considered stable when its levels were within ± 15 % of the baseline, they provided experimental evidence that 17OHP did not significantly change for 1 year even at room temperature, thus being considered stable.

In summary, six of the previously discussed investigations evaluated the stability of different markers in the short-term (1 month) (5,9-12,14), five in the medium-term (1 to 2 years) [6,8,13,15,17], and two in the long-term (12 and 15 years) [7,16]. Three investigations conducted their studies using newborn DBS specimens [6,7,16] but in all cases samples corresponded to healthy or screen-negative newborns. Nine studies evaluated 3 or more storage temperature conditions, but all included at least one condition corresponding to room or ambient temperature. Four investigations conducted part of their evaluations in environments of high humidity [5,9,11,14], one stated desiccants were not used (8), three did not describe their humidity conditions being supposed that they were ambient humidity [12,16–17], and the remaining five worked in controlled conditions [6,7,10,13,15]. These diverse scenarios highlight the difficulties faced when an objective comparison is attempted between the results of previously published experiences and the results of this paper.

The investigations cited demonstrated the detrimental effects of high humidity on analyte stability and specimen integrity, and that storage at low and controlled temperatures improves analyte recovery. Similar findings also were demonstrated by our own study.

Since weather in La Plata is characterized by a high monthly average humidity throughout the year and one third of the year presents average monthly maximum temperatures > 25 °C, it was inferred that analyte recovery would be moderately to highly reduced in DBS specimens stored in suboptimal and uncontrolled conditions.

Our experience showed predictable decreases in recovery for Phe, TSH and IRT as a function of the storage time in uncontrolled conditions using linear, logarithmic and polynomial adjustments respectively, which allowed for estimating the expected analytes degradation.

The comparison of Phe recovery results regarding the two longest previously published evaluations showed similar results, even notwithstanding some differences in storage conditions. In fact, Phe recoveries estimated from our linear trend curve were 31 and 64 % at 15 and 2 years of storage, which agree with recoveries reported by Strnadová *et al.* [7] at room temperature in a dry environment (41 %), and Prentice *et al.* [8] at 21 °C without desiccants (60 %) along the same storage periods, respectively.

When evaluating TSH stability, and similarly to those described for several authors, our results showed a poor stability resulting from a fast degradation rate, strongly influenced by the adverse humidity conditions. In quantitative terms, the estimated TSH recovery obtained from our logarithmic trend curve at 1 year of storage was similar (47.3 %) to those reported by Magalhães *et al.* (47.7 %) [13].

Regarding IRT, Li *et al.* [15] reported a 25 % of concentration reduction when stored during 1 year at room temperature (25 °C) with desiccants, while the estimated IRT reduction obtained from our order-5 polynomial trend curve at 1 year of storage was lower (40 %), demonstrating an increased IRT degradation in our study probably related to our more adverse storage humidity conditions.

A comparative evaluation of our Phe, TSH and IRT results, showed a clear difference in the experimental Phe behavior in terms of stability and recoveries, with it being worthwhile to note that Phe was more stable and less affected by the adverse temperature and humidity storage conditions. Instead, TSH and IRT were more sensitive to uncontrolled conditions, probably due to the fact that besides the progressive analyte instability, other factors that may affect the elution efficiency come into play, such as molecular size and protein fixation to the filter paper matrix.

In contrast, 17OHP and TGal showed more random recoveries which did not allow for applying any kind of mathematical function to look for trends.

Despite these considerations, our results suggest that 17OHP seems to be more stable than TSH and IRT, as supported by our observation that DBS specimens stored for 2 years in uncontrolled conditions presented recoveries > 71 % (n = 11). Previously, Murphy *et al.* [6] and Grecsó *et al.* [17] stated that 17OHP was considered stable for 1 year, and Török *et al.* [16]

reported a 17OHP decrease of 3.2 % per year and concluded it can reliably be used for retrospective examinations when stored for more than a decade. Additionally, our 17OHP results showed different recovery behavior at high concentrations (451.0 – 995.0 nmol/L) compared to what was observed at lower 17OHP levels.

Regarding TGal, our research did not find a relationship between storage conditions and analyte recovery. TGal measures the content of free galactose (Gal) and galactose-1-phosphate (Gal-1P) present in the sample from previous hydrolysis of Gal-1P. GALK, GALT and GALE deficiencies are characterized by the increase of Gal; Gal plus Gal-1P; and Gal, Gal-1P plus UDP-Gal, respectively, thus probably explaining the differences in recoveries observed between the deficiencies. In addition, recoveries above 100 % were found in GALE and GALT deficiencies, evidencing a possible liberation of Gal from other sources such as UDP-Gal in the first case, and from galactosylated macromolecules in both. However, this last hypothesis does not explain why specimens with GALK deficiencies presented so very low recoveries when DBS were stored in uncontrolled conditions, and showed recoveries of about 65 % when stored at -20 °C.

In spite of this, TGal seems to be a relatively more stable analyte than the others when stored in uncontrolled conditions, given that 64 % of specimens presented recoveries ≥ 85 % irrespective of the storage time. These findings cannot be corroborated with other experiences because fewer stability studies have been published for TGal.

As far as we know this is the first stability study that evaluated analytes markers in DBS specimens from newborns diagnosed with PKU, CH, CF, CAH and Galactosemia, in which specimens were stored in suboptimal and uncontrolled storage conditions of temperature and humidity during a long-term period up to 23 years.

Our research has two limitations in experimental design: the residual DBS specimens were retested in only one run in singlicate, and the magnitude of the severity of the uncontrolled storage conditions could not be quantified.

The strengths of this investigation include using actual DBS specimens from newborns diagnosed with the five mentioned disorders, thus determining that concentrations of the measured analytes were naturally increased in each specimen and were not the result of an external enrichment. Besides, the study was conducted using a large number of samples compared with other studies and covered more than 20 years of storage time. Furthermore, the inherent variation attributable to methods performance was minimized because initial testing and retesting were made, in most cases, using the same method or analytical principle.

Although the need to retest a specimen to discard a false negative result in residual DBS specimens stored in the very long-term would be rare, our results make an important contribution to the knowledge about the period that some analytes could maintain abnormal results during storage in uncontrolled conditions of temperature and humidity, as showed by the summary presented in Table 4.

This information is valuable for NBS programs in developing countries, and elsewhere, where the economic limitations do not allow for residual DBS specimens storage in optimal conditions of temperature and humidity.

Table 4. Percentage of abnormal results at retesting found at different analytes concentrations and storage periods.

Analyte	Concentration at initial testing	Storage time	Percentage of abnormal results
Phe	≥ 6.0 mg/dL	≤ 6 years	100 %
	≥ 6.0 mg/dL	≤ 16 years	90 %
TSH	≥ 55.7 µU/mL	≤ 3 year	100 %
	≥ 229.2 µU/mL	≤ 12 years	100 %
IRT	≥ 145.5 ng/mL	≤ 4 years	86 %
17OHP	≥ 22.9 ng/mL	≤ 21 years	84 %
	≥ 74.5 ng/mL	≤ 21 years	99 %
TGal †	≥ 9.3 mg/dL	≤ 13 years	100 %

† Valid only for severe GALT deficiencies and GALE deficiencies.

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Authors' Contributions

VVD Specimens selection, technical procedures and data acquisition, discussion and revision. GJCB Conceptualization, methodology design, drafting, data analysis and interpretation, manuscript writing, plots elaboration, discussion, revision and editing.

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