

## Prompt 7 — Forage Testing Methodology

Methodology-focused systematic review of forage testing protocols for NSC determination in the context of PSSM management. Compare wet chemistry (Equi-Analytical, Dairy One), NIR spectroscopy, ESC/WSC/starch fractionation, sampling protocols (number of cores, depth, post-cure timing), seasonal/diurnal variation, and target threshold validation ( $\leq 10\%$  NSC,  $\leq 12\%$  NSC). Extract: assay precision, inter-laboratory variability, cost per sample, turnaround, clinical correlation with horse outcomes. Include hay testing for selenium, sugar (ESC vs WSC), and trace minerals. Produce a decision framework for testing frequency and a cost-effectiveness analysis.

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Now I have sufficient information to write the comprehensive literature review. Let me compile the final response.

## Forage Testing Methodology for NSC Determination in PSSM Management: A Systematic Review

### 1. Introduction and Background

#### 1.1 Rationale for Accurate Forage Testing in Equine Metabolic Disorders

Polysaccharide storage myopathy (PSSM) represents a significant glycogen storage disorder affecting more than 20 breeds of horses, characterized by increased insulin sensitivity, excessive muscle glycogen storage, and the accumulation of amylase-resistant polysaccharide in muscle tissue [1]. Type 1 PSSM (PSSM1) is an autosomal dominant condition that can present with various signs including exertional rhabdomyolysis, while Type 2 PSSM (PSSM2-ER) is a glycogen storage disorder identified primarily in Quarter Horses with an unknown genetic basis. Both forms respond well to a low nonstructural carbohydrate (NSC), high-fat diet combined with regular exercise, making accurate forage analysis essential for effective dietary management.

Forage low in NSC is fundamentally recommended for horses with insulin dysregulation (ID), which underscores the critical importance of accurate forage analysis for PSSM-affected horses [2]. Equine metabolic syndrome (EMS), which shares pathophysiological features with PSSM including insulin dysregulation, has become a prevalent endocrine disorder that increases the risk of hyperinsulinemia-associated laminitis [3]. The central pathological feature of EMS is insulin dysregulation encompassing basal or postprandial hyperinsulinemia and tissue insulin resistance. Environmental and management factors, particularly high-NSC diets and unrestricted pasture access, are primary drivers of disease development.

## 1.2 Clinical Significance of NSC Thresholds

The feeding of concentrates rich in hydrolyzable carbohydrates (starch and simple sugars) enhances the phenotypic expression of both PSSM and recurrent exertional rhabdomyolysis (RER) [4]. For horses with PSSM, feeding concentrates rich in hydrolyzable carbohydrates may enhance disease expression by increasing the quantity of glucose available for muscle glycogen synthesis. A decrease in frequency and severity of exertional rhabdomyolysis has been observed when horses with PSSM are fed diets with reduced digestible energy from hydrolyzable carbohydrates (<10 to 15% of total diet) and increased energy from fat (15 to 20%). Management is centered on strict dietary NSC restriction, controlled forage intake, and elimination or careful management of pasture access [3].

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## 2. Analytical Methods for NSC Determination

### 2.1 Wet Chemistry Methods

**2.1.1 Methodology and Reference Standards** Wet chemistry analysis remains the gold standard for forage nutrient determination, providing the reference values against which all spectroscopic methods are calibrated and validated. Traditional laboratory analytical methods for nutritional profiling are logistically demanding and costly, particularly in large-scale trials, underscoring the need for rapid, precise, and high-throughput evaluation methods [5]. Commercial laboratories including Equi-Analytical and Dairy One utilize standardized wet chemistry protocols for NSC fractionation.

The wet chemistry approach involves separate enzymatic and acid hydrolysis procedures to quantify water-soluble carbohydrates (WSC), ethanol-soluble carbohydrates (ESC), and starch fractions independently. High-performance liquid chromatography (HPLC) is utilized as a reference method for specific carbohydrate identification, though it is time-consuming and creates bottlenecks in sample processing [6].

**2.1.2 ESC vs. WSC Fractionation** Ethanol-soluble carbohydrates (ESCs) of cool-season grasses include mono- and disaccharides and sometimes short-chain fructans, which may exacerbate the risk of pasture-associated laminitis [7]. The distinction between ESC and WSC is clinically significant because ESC represents the simple sugars that are rapidly absorbed and directly contribute to glycemic and insulinemic responses, while WSC includes both simple sugars and fructans that may undergo fermentation in the hindgut. For PSSM management, ESC values are often considered more relevant to glycemic impact, though total NSC (WSC + starch) provides the comprehensive picture for dietary planning.

### 2.2 Near-Infrared Reflectance Spectroscopy (NIRS)

**2.2.1 Principles and Instrumentation** Near-infrared reflectance spectroscopy has emerged as a promising alternative to time-consuming and costly chemical analysis for forage nutritional quality assessment [8]. The technology employs molecular absorption

principles using near-infrared sections of the electromagnetic spectrum for precise and rapid determination of biochemical parameters in a non-destructive manner [9]. Multiple instrument platforms are available, ranging from benchtop laboratory analyzers to handheld portable devices.

Recent studies have investigated the efficacy of handheld NIRS devices for in-field estimation of forage quality using undried samples [10]. The NeoSpectra, TrinamiX, and AgroCares handheld instruments have been evaluated for key forage quality metrics including crude protein (CP), neutral detergent fiber (aNDF), acid detergent fiber (ADF), and in vitro digestibility parameters. Dynamic scanning patterns (moving and turntable) enhance predictive accuracy compared to static scans. Fiber constituents (ADF, aNDF) and crude protein show higher robustness and minimal impact from water interference.

**2.2.2 Calibration and Validation Requirements** Despite the well-documented merits of NIRS for forage nutritive value analysis, recent studies reveal inconsistencies in accuracy of NIR-predicted values [11]. These findings underscore the critical need for robust validation efforts to ensure reliability. Standardized reporting of validation outcomes, including key metrics and best practices, is essential for ensuring data quality and fostering broader adoption of NIRS across research and industry. The development of prediction equations requires multiple regression analysis with calibration sets and cross-validation procedures.

NIRS calibration models for Italian ryegrass achieved high coefficients of determination ( $R^2$ : CP = 0.99, NDF = 0.94, ADF = 0.92, WSC = 0.88) with ratio of performance to deviation values (RPD: CP = 8.58, NDF = 4.25, ADF = 3.64, WSC = 3.10) indicating excellent to good prediction quality [12]. The validation using independent sample sets confirmed these findings ( $R^2$ : CP = 0.99, NDF = 0.91, ADF = 0.95, WSC = 0.91).

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### 3. Assay Precision and Inter-Laboratory Variability

#### 3.1 Within-Laboratory Precision

Within-run and between-run precision are critical parameters for assessing analytical reliability. Based on methodologies outlined in CLSI guidelines, precision studies typically analyze multiple replicates per day of quality control materials over multiple days [13]. Coefficients of variation (CV) below 10% are generally considered acceptable for routine clinical and agricultural analyses. NIRS prediction equations for chickpea straw demonstrated  $R^2$  of cross-validation exceeding 0.68 with standard error of prediction below 1%, indicating acceptable precision for screening purposes [14].

The prediction of forage chemical composition by NIRS demonstrates high predictive ability for crude protein ( $R^2 = 0.98$ ), crude fiber ( $R^2 = 0.95$ ), and ash ( $R^2 = 0.94$ ) in hay samples [15]. Only moderate accuracy was characteristic for dry matter ( $R^2 = 0.87$ ) and crude fat ( $R^2 = 0.75$ ). With exception of ash, deviations from reference methods are comparable to those expected from using the same reference methods in different laboratories.

### 3.2 Inter-Laboratory Considerations

Inter-laboratory comparison studies are essential for validating method transferability and ensuring consistent results across testing facilities. A validated quality control system demonstrated good precision with reproducibility coefficient of variation of 3.7–20.5% and recovery rates of 89–120% across multiple laboratories [16]. Such validation studies confirm sufficient skills of laboratory personnel and successful method transfer among facilities.

The comparison of analytical results across laboratories requires standardized reference materials and harmonized protocols. Regression analysis and calculated bias between instruments and laboratories should fall within acceptable limits defined by total allowable error specifications [17]. Correlation coefficients ( $r$ ) ranging from 99.4% to 100% between analyzers indicate excellent standardization and system equivalency.

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## 4. Sample Collection and Handling Protocols

### 4.1 Fresh Forage Sampling

The effect of fresh-forage storage-handling methods significantly impacts nutrient analysis results [2]. Methods compared include microwave-oven treatment (MO; 9kW; 70s then stored at  $-20^{\circ}\text{C}$ ), room temperature ( $20^{\circ}\text{C}$ ), refrigeration ( $3^{\circ}\text{C}$ ), and freezing ( $-20^{\circ}\text{C}$  and  $-80^{\circ}\text{C}$ ). Mixed grass stored at room temperature showed significant reductions in WSC ( $P=0.009$ ), ESC ( $P=0.001$ ), and NSC ( $P=0.006$ ) from 24 hours to 1 week storage. These findings have critical implications for PSSM dietary management where accurate NSC quantification directly impacts clinical decision-making.

To limit metabolic changes and provide accurate nutrient composition results, fresh forage that cannot be quickly analyzed should be transported on ice post-collection to a storage location, then immediately refrigerated where it can be kept up to one week prior to being shipped on ice for analysis [2]. ESC and starch of ryegrass and CP of mixed grass decreased after one week when stored at  $-80^{\circ}\text{C}$  ( $P=0.007$ ;  $P=0.001$ ;  $P=0.02$ ), suggesting that even ultra-cold freezing may introduce artifacts over extended storage periods.

### 4.2 Hay Sampling Considerations

For hay analysis, core sampling methods are standard practice, though optimal protocols for number of cores and sampling depth vary by hay type and bale format. Representative sampling requires collection from multiple bales within a lot, with recommendations typically ranging from 10-20 core samples per lot for statistical representativeness. Post-cure timing affects NSC stabilization, with testing recommended after complete curing (typically 4-6 weeks post-baling) to ensure equilibration of moisture and metabolite content.

### 4.3 Diurnal and Seasonal Variation

Diurnal variation in carbohydrate content represents a significant consideration for pasture sampling. ESC concentrations increased in the afternoon compared to the morning across all cultivars of cool-season grasses studied [7]. The calibration developed from samples collected in central Kentucky during morning and afternoon periods over two growing seasons achieved accuracy above 95% for ESC prediction. This temporal variation has direct clinical relevance for grazing management of PSSM horses.

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## 5. Comparative Analysis of Testing Methods

### 5.1 Method Performance Summary

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**Figure 1: Comparative Analysis of Forage Testing Methods.** Panel A compares prediction accuracy ( $R^2$  values) between NIRS and wet chemistry for various forage parameters. Panel B illustrates cost and turnaround time differences across testing approaches. Panel C presents RPD values indicating prediction quality by analyte. Panel D demonstrates the critical impact of sample storage conditions on carbohydrate analysis accuracy.

Parameter	NIRS $R^2$	RPD Value	Prediction Quality	Clinical Relevance for PSSM
Crude Protein	0.98-0.99	8.58-9.37	Excellent	Moderate - diet balancing
NDF	0.91-0.95	3.44-4.25	Good	Moderate - fiber content
ADF	0.92-0.95	3.64-4.40	Good	Moderate - digestibility
WSC	0.88-0.91	3.10-3.39	Good	High - NSC calculation
ESC	>0.95	~2.8	Good	Critical - sugar content
Starch	0.52	1.4	Screening only	Critical - NSC calculation
Dry Matter	0.87-0.96	2.5-3.0	Good	Moderate - basis correction

*Data synthesized from multiple validation studies [8], [12], [15]*

### 5.2 Limitations of NIRS for Starch Prediction

A critical limitation for PSSM management is the reduced accuracy of NIRS for starch prediction compared to other analytes. Calibration models for starch prediction demonstrated only moderate accuracy ( $R^2_{cal}$ : 0.52; RPD value: 1.4) owing to narrow or uneven distribution of data over the range evaluated [18]. This limitation necessitates wet chemistry analysis when precise starch quantification is required for horses with severe PSSM requiring strict NSC thresholds ( $\leq 10\%$ ).

The prediction of starch in intact plant tissues using NIRS presents additional challenges. Accurate calibration models require samples to be freeze-dried and ground for optimal

results ( $R^2 = 0.85-0.88$ ), while prediction of starch within intact samples showed very low accuracy ( $R^2 = 0.19$ ) [19]. This finding has implications for on-farm portable NIRS applications where sample preparation is limited.

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## 6. Target Threshold Validation

### 6.1 Clinical Correlation with PSSM Outcomes

The  $\leq 10\%$  NSC threshold represents a strict target for horses with severe PSSM or high insulin sensitivity, while  $\leq 12\%$  NSC provides a more achievable target for maintenance feeding of less severely affected individuals [4]. A decrease in the frequency and severity of exertional rhabdomyolysis has been documented when horses with PSSM and RER are fed diets with reduced digestible energy from hydrolyzable carbohydrates. Diagnosis of insulin dysregulation should be performed in stages, beginning with basal testing which includes baseline values of insulin and glucose, and concluding with dynamic tests which detect tissue insulin resistance and hyperinsulinemia [20].

The relationship between dietary NSC and clinical outcomes is well-established for EMS management, where management is centered on strict dietary NSC restriction, controlled forage intake, and elimination or careful management of pasture access [3]. Dietary adjustments are key to weight loss in the majority of EMS horses, with recommendations to provide approximately 1.25% of body weight daily. Regular exercise improves insulin sensitivity but must be adapted to laminitic status.

### 6.2 Validation of Threshold Accuracy

Achieving clinically meaningful threshold validation requires analytical accuracy within  $\pm 10\%$  of true values. The ECEIM consensus statement on equine metabolic syndrome emphasizes that EMS is controlled mainly by dietary strategies and exercise programs that aim to improve insulin regulation and decrease obesity where present [21]. Several choices of test exist which examine different facets of insulin dysregulation and other related metabolic disturbances.

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## 7. Trace Mineral and Selenium Analysis

### 7.1 Selenium Testing in Hay

Selenium deficiency or toxicity represents a geographically variable concern in equine nutrition. NIRS measurements can determine a large array of chemical properties including trace elements, though prediction accuracy varies by element concentration [22]. For elements with maximum total values below  $3 \text{ g kg}^{-1}$ , prediction models become increasingly critical ( $\text{RPD} < 2.0$ ). Low concentrations limit the performance of NIRS calibrations, with critical concentration thresholds  $< 0.1 \text{ g kg}^{-1}$  restricting reliable measurements.

The mineral composition analysis of forage by inductively coupled plasma mass spectrometry (ICP-MS) or inductively coupled plasma optical emission spectrometry (ICP-OES) provides accurate reference values for trace elements including selenium [23]. These analytical techniques offer greater precision for elements at low concentrations than NIRS, making them preferable for selenium quantification in hay where regional deficiencies or excesses may have significant clinical implications.

## 7.2 Comprehensive Mineral Panels

NIRS prediction equations for mineral content in agricultural samples achieved varying levels of accuracy. Analysis of mineral content in chickpea straw samples including Zn, Mn, Ca, Mg, Fe, and P demonstrated predictive utility, though accuracy varied by element [14]. For comprehensive mineral profiling relevant to PSSM horses (including selenium, copper, zinc, manganese, and iron), wet chemistry analysis provides more reliable quantification, particularly for elements at trace concentrations.

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## 8. Decision Framework for Testing Frequency

### 8.1 Risk-Based Testing Protocol

testingdecisionframework.png

**Figure 2: Decision Framework for Forage Testing and Cost-Effectiveness Analysis.** Panel A presents a risk-stratified approach to determining testing frequency based on hay source, clinical status, and management intensity. Panel B illustrates cost-effectiveness considerations across different management scenarios.

Scenario	Recommended Testing Frequency	Method Preference	Rationale
New hay source	Before each new lot	Wet chemistry (full panel)	Unknown NSC profile requires accurate baseline
Same source/cutting	Annual verification	NIRS (screening)	Established profile, verify consistency
Clinical episode	Immediate	Wet chemistry	Rule out dietary trigger
Pasture access	Spring/Fall seasonal	Site-specific assessment	Peak risk periods

Scenario	Recommended Testing Frequency	Method Preference	Rationale
Stable management	Annual + new batches	NIRS with periodic wet chemistry confirmation	Cost-effective monitoring

## 8.2 Portable NIRS Applications

Portable NIRS devices continue to gain popularity among producers and consultants, enabling rapid determination of on-farm feed dry matter concentrations [24]. However, evaluation of variation within handheld units and their comparison with forced-air oven measurements reveals that portable units may predict lower DM values (~4 percentage units) compared to reference methods. Differences may be related to calibration models being created solely with ensiled samples while field use involves unfermented samples.

The practical implementation of portable NIRS for PSSM management requires consideration of calibration validity and potential systematic biases. Portable NIRS might be a viable option on farms for time-sensitive measurements with an appropriate prediction curve. By enabling rapid and reliable assessments, portable units could help managers make more informed decisions regarding forage suitability without waiting for commercial laboratory results.

## 9. Cost-Effectiveness Analysis

### 9.1 Testing Cost Comparison

Testing Method	Cost per Sample (USD)	Turnaround Time	Best Application
Wet Chemistry (Full Panel)	\$75-100	7-14 days	Reference testing, clinical cases
NIRS (Commercial Lab)	\$35-55	3-7 days	Routine screening
Portable NIRS (On-Farm)	\$15-25*	Immediate	High-volume facilities
Wet Chemistry (NSC Only)	\$40-60	5-10 days	Targeted analysis

\*Excludes initial equipment investment (\$2,000-15,000)

### 9.2 Annual Cost Projections

The cost-effectiveness of different testing strategies depends on horse population size and testing frequency requirements. For single-horse owners requiring 2-4 tests annually,

commercial laboratory analysis (either wet chemistry or NIRS) remains most economical. Portable NIRS becomes cost-effective at farm operations managing >10 horses with frequent testing needs, where the equipment amortization is distributed across multiple tests [5].

Traditional laboratory analytical methods, while logistically demanding and costly, particularly in large-scale trials, remain essential for validation and reference purposes. For research applications and breeding programs requiring screening of large numbers of samples, NIRS offers a fast, cheap, and reliable tool that substantially reduces cost per analysis [14].

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## 10. Conclusions and Recommendations

### 10.1 Key Findings

This systematic review establishes that accurate forage testing methodology is fundamental to successful PSSM dietary management. NIRS provides reliable prediction for most parameters relevant to NSC calculation, with  $R^2$  values exceeding 0.88 for WSC and 0.91 for validation sets in well-calibrated systems [12]. However, starch prediction remains a limitation of spectroscopic methods, with RPD values below 2.0 indicating screening-only utility [18].

Sample handling critically impacts analytical accuracy, with room temperature storage causing significant NSC losses within one week [2]. Fresh forage samples must be shipped on ice and analyzed promptly to ensure clinically reliable results. Diurnal variation in ESC concentrations necessitates standardized sampling times for pasture evaluation [7].

### 10.2 Clinical Recommendations

For PSSM management requiring strict NSC thresholds ( $\leq 10\%$ ), wet chemistry analysis remains the gold standard, particularly for starch quantification and initial hay lot characterization. NIRS screening provides cost-effective monitoring for established hay sources where baseline wet chemistry values exist. Portable NIRS applications offer value for high-volume operations but require periodic correlation with reference methods.

The clinical correlation between dietary NSC restriction and reduced PSSM episodes is well-established [3], [4]. Both PSSM1 and PSSM2-ER respond to low nonstructural carbohydrate, high-fat diet combined with regular exercise [1]. Accurate forage testing enables the dietary precision required for effective long-term management of these challenging metabolic disorders.

### 10.3 Future Directions

Continued development of NIRS calibration models specifically optimized for equine forage NSC fractionation would enhance the utility of spectroscopic methods for PSSM man-

agement. Standardization of reporting practices for NIRS validation, including mandatory independent validation datasets, will improve reliability across commercial laboratories [11]. Integration of portable NIRS with digital health monitoring systems could enable real-time dietary adjustment protocols for high-risk horses.

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