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Development and Validation of Proximal Chemistry Analytical Procedure in Semi-automated Equipment

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Abstract: The proximal chemical analysis (AQP) includes 5 fundamental tests, which are: determination of crude protein, determination of crude fiber, determination of humidity, determination of ashes and determination of fat. This last determination can be made in two different ways, which will depend on the type of sample being treated, as well as the amount of fat expected to be obtained in the food to be analyzed. For foods with low amounts of fat the hydrolysis technique is used, which is divided into 3 phases. All the methods before being taken to the daily practice in a laboratory of food analysis either internal control, verification or third authorized must be validated, in order to obtain consistent, robust and reliable results. In those cases in which the method that will be tested differs with the method that is reported in the literature, a comparison of both methods should be made in order to ensure that both are compatible and the results will be equally reliable. In the validation, the acceptance parameters will be established for each one of the tests that are carried out in it, while at the end of it the acceptance criteria for the general method will be established. The objective of this work was to carry out the development of analytical methodology that was validatable in order to reduce analysis time by using semi-automated equipment. In the case of semi-automated equipment, this comparison of methods is carried out, as it was the case of the analysis of fat with hydrolysis, which used a hydrolysis unit and the extraction equipment using samples of finished food for animal consumption. The results obtained in the validation using the traditional method correspond to a CV less than 2%, while the results obtained using semi-automated equipment correspond to a CV less than 2% for the case of fat determination with hydrolysis.

Key words: Validation, analytical methods, semiautomatic, animal feed.

1. Introduction

The analyses are based fundamentally on physical, chemical and physicochemical principles; for the specific case of food products it is required to know some aspects of the biology of it, since most of the foods for animal consumption are of vegetable origin, that is, they are mixtures of corn, soy, wheat, barley, distillery grains (DDg's), among others and for that reason it is required to know the nature of the ingredients used for the preparation of the food [1, 2].

The proximal chemical analysis (PCA) is used in the ingredients that are intended to formulate a diet as well as in finished foods, providing a control, which verifies that they meet the specifications established during the formulation. These analyses will indicate the content of moisture, ash, crude protein or total nitrogen, fat, crude fiber, and in some cases they are important to determine urea, among others. Knowing the results in the concentrations that each analysis gives us is of the utmost importance, since when concentrations are present, either high or low, some of the ingredients can cause negative side effects in animals [2].

Within the laboratory, the analytical development of a method begins with the need of a client, since when submitting a new product to production it will require specifications that will be delivered to the corresponding authority, for which the pertinent

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analyses must be carried out. The first step is to know not only the formulation, but also to know the main parameters to be analyzed, followed by a search in the recognized literature, as is the case of the AOAC methods (Official Association of Agricultural Chemists, by its initials in English), NMX's (Mexican Standards), NOM's (Official Mexican Standards) [1, 3].

Because the validation is a documentary evidence of the data obtained from the tests carried out, they must demonstrate functionality, consistency and robustness. That is the reason why the realization of the validation of analytical methods, is a fundamental part of the Quality Management System (QMS) of every company. The validation also allows ensuring that the analysis of foods for animal consumption, is made following compliance with current legislation and regulations, as well as what is established in the Validation Master Plan (VMP), in order that the method used meets the requirements for its application within the laboratory [1, 3-5].

The validation of analytical methods leads to decreasing non-conformities, which gives reliability of the results, meets the established quality standards.

To carry out the validation of an analytical method, it is important to use a matrix or a test that demonstrates the application of this method. The characteristics with which they must comply with the matrix with which the method is validated are the following (these are the most representative, however, they are not limiting):

- which has greater knowledge
- the most complex.
- the most requested.
- the most representative of a food group: dairy products, meat products, fish products, cereals, fruits, vegetables, bread products, honey, confectionery products, spices, among others.
- the most representative representation by pharmaceutical form: capsule, lozenge, tablet, suspension, syrup, ointment, eye drops, foam, gel, suppository, patch, jelly, ovule, among others.
- short half life time.
- according to the speed.
- chemical composition of the sample.
- physical state.
- homogeneity of the sample.
- worst case.

The introduction of semi-automated equipment in the food industry, specifically those used to carry out the proximal chemical analysis, has brought great advantages over methods that are performed in a traditional manner. The main advantages are: decrease in analysis time, lower energy consumption and reagents, more versatile analysts, reduction in waste production, among others [4, 6-8].

The means for the development of an analytical method and the use of the validation was carried out with the traditional method of the quantification of the fat with the hydrolysis with the method that is carried out in semi-automated equipment.

The determination of the fat requiring hydrolysis was carried out in three stages, the first stage in dehydrolysis, where an Opsis Liquidline equipment was used; the second stage is a drying of the sample and the third stage is extraction, where the Soxtec equipment (Foss Tecator) was used [9-11].

2. Materials and Methods

2.1 Equipment

Analytical balance (Ohaus, AP21OS)
Filtration bag (Opsis, SX110-A-1004)
Filter holder (Opsis, SX110-A-1004)
Acrylic rack (Opsis, SX110-A-1004)
Recirculator MR20 (Lauda, MR 20)
HydROC Hydrolysis Unit (Opsis, SX110-A-1004)
Oven (Felisa, 242A)
Spray system (Foss)
Soxtec (Foss tecator, 1983)
Aluminum cups (Foss)

2.2 Reagents

Hydrochloric acid solution 37, 40 and 33% (v/v)

Distilled water

Animal food samples (made mostly of corn)

2.3 Methods

Methods established by the AOAC were used; the techniques were implemented according to the type of equipment present within the laboratory.

In the determination of fat, a Soxtec semiautomatic device (Foss Tecator) was used, and for the samples that require hydrolysis, the Hydrolysis Unit (Opsis Liquidline) was used; Its principle is similar to that of a Soxhlet system that consists of five phases: sample preparation, hydrolysis, drying of the sample, extraction and drying of the ethereal extract.

The first phase in the preparation of the sample, in which a bibliographic study can be carried out to determine the quantity of samples that are going to be used, the type of solvent that will be used during the extraction, the time needed in each phase, likewise it will be known if the sample is going to need a previous treatment before the analysis, in other words a pre-extraction.

The second phase is to carry out the hydrolysis, for the case of a finished food sample of animal consumption, acid hydrolysis is carried out; however there are other matrices that are treated with basic hydrolysis. In this phase the sample is placed into filter cartridges, then into containers inside the equipment, the equipment is turned on and acid reflux begins. Once the reflux time has elapsed, the acid solution can be drained and washed with water to obtain an almost neutral or neutral pH.

The third step is to perform a drying of the sample, under the conditions that all the matrices require to be analyzed; knowing that some of them can be degraded at high temperatures.

The fourth phase is to place the samples in the Soxtec equipment (Foss Tecator), as well as the aluminum cups at constant weight with the solvent, which is specific for each sample; however the most used dissolvent is petroleum ether. At this point, a plate under the aluminum cups will heat the dissolvent to boiling point, then a rinse is performed and finally the sample is recovered.

As a final stage we have the drying of the samples, in this stage the aluminum cups are removed from the equipment and the excess dissolved is removed as well with a drying oven, the cups are then placed in a desiccator, weighed and the percentage of fat contained in the samples is calculated.

Finally, the comparison of the method is made using semi-automated equipment against the traditional method. In the traditional method, as is the case of Soxhlet, compared to the semi-atomized, and doing the hydrolysis, it consists in the samples getting into flasks, together with a filtering aid, boiling bodies to which an acid solution is added, they undergo into reflux, and at the end of it the samples are filtered through a filter paper with the help of a funnel, washed with warm water until a pH close to neutrality is reached, then the flasks are perfectly cleaned with solvent to avoid residues of the sample in the flask. Once the whole sample is in the filter paper, it is taken to a stove so that the sample remains dry, and it is possible to proceed for the extraction of fat following the Soxhlet method that consists of placing the sample in the presence of solvent which will take 3 phases that are boiling, rinsing and solvent recovery. Finally, the excess solvent is removed from the extraction vessels containing the fat, to be later weighed and calculate the fat content [10-12].

In order to carry out this analysis, the validation was carried out to verify that both analytical methods had reliable results and that these would fall within the acceptance criteria for each parameter. Table 1 shows evaluated parameters, the acceptance criteria, how to prepare the samples for each parameter and some recommendations on how to carry out the analysis.

3. Results

The semi-automated method was compared with the traditional method, in different validation parameters;

Table 1 Evaluated parameters during validation, characteristics and acceptation criteria.

Validation elements	Samples	Determinate	Recomendations	Acceptation criteria
Accuracy	Prepare independently 6 samples corresponding to 100%	$\bar{Y}$, S y CV	Samples can be prepared from the same standard solution.	CV ≤ 1.5% for physicochemical methods CV ≤ 2% for physicochemical methods
Linearity	Prepare independently 5 samples or triplicate dilutions at different concentration levels	m, b, r^2 IC(β1) *t* Student. Plot the concentration graph VS analytical response	The concentration levels should be equally spaced in the range of interest	$r^2 \geq 0.98$ IC (β1) should not include 0
Specificity	Prepare independently 3 samples corresponding to 100%	r^2, m, b, IC (μ) % of recovery CV % of recovery	The concentration levels should be equally spaced in the interest range	IC (μ) 97-103% spectrophotometric or chemical methods CV % of recovery less than 3% spectrophotometric or chemical methods
Repeatability	Prepare independently 3 different concentration levels or triplicate dilutions at different concentration levels per sixfold	$\bar{Y}$, S, CV IC (μ) % of recovery CV % of recovery	The concentration levels should be equally spaced in the interest range It is determined with a single analyst using the same instruments and methods	IC (μ) 97-103% spectrophotometric or chemical methods CV % of recovery less than 3% spectrophotometric or chemical methods
Intermediate precision	Prepare independently 3 samples corresponding to 100%	$\bar{Y}$, S, CV *F* of Fisher	It is determinated at the same laboratory by different analysts on different days	CV ≤ 3% to spectrophotometric or chemical methods
Robustness	Under normal and operating conditions, analyze the sample in triplicate	|di|	Internal factors of method	|di|≤ 3% to spectrophotometric or chemical methods

a partial validation was carried out, since the method was obtained from both the AOAC as well as the NMX.

The precision of an analytical method is determined by analyzing a sufficient number of repetitions of a homogeneous sample, this allows a statistically valid calculation of the relative standard deviation (coefficient of variation). To evaluate this parameter, an analyst prepared 6 samples of animal feed independently. The samples were subjected to the fat procedure with hydrolysis described above and obtained a coefficient of variation (CV) of 0.92 for the semi-automated method [11-13].

In the case of intermediate precision, the variation was evaluated when the analytical method was carried out by two different analysts (A and B) independently. On different days, they prepared 3 samples each, the process was carried out using semi-automated equipment (Hydrolysis unit, Opsis Liquidline and Soxtec fat extraction equipment, Foss Tecator). The result obtained by analyst A showed a coefficient of variation CV of 1.97 while analyst B obtained a CV of 1.62, later on different days both analysts performed the same procedure obtaining a CV for analyst A of 1.62 and for analyst B of 1.82. A statistical analysis of variance (ANOVA) was performed (shown in Table 2) where the results obtained by the different analysts (A and B) and the results obtained in different days were evaluated [11, 13].

In terms of reproducibility, it can be evaluated using a minimum of nine determinations of samples of known composition with a concentration that covers the interval specified for the method, in this study an analyst was prepared independently in triplicate and in

3 different levels of concentration (high, medium and low) by a known matrix. That is, for this particular case, instead of concentrations, 3 different weights were used, which were evaluated under the same conditions, with the purpose of knowing which of them was the optimal one to carry out the determination. The CV for a low, medium and high weight was 15.86, 1.97 and 1.59 respectively. It was determined that using a medium weight or a high weight is optimal to carry out this type of determinations, however, using a lower weight would result in unreliable results in the determination, since the CV in this analysis was higher than the one reported in the acceptance criteria. Later, an ANOVA analysis of variance was carried out, obtaining the results shown in Table 3 [12, 13].

The robustness shows that there is no influence of the operational and environmental variables on the results of the analysis, it is determined using different operating and environmental conditions, but within the established parameters. Within this parameter an analyst independently is prepared by triplicate 3 different levels of concentration (high, medium and low). That is, 3 levels of concentration of the acid solution were used, these levels are reported both in the NMX and in the application notes, which are part of the semiautomatic equipment, in the same way they are found as support to develop the analyzes and they are based on AOAC methodologies [11, 12].

The samples were subjected to the same treatment, with the same analyst, obtaining a CV of 1.69 for the lowest concentration; the CV was 1.97 for the median concentration and 2.09 for the highest concentration. When obtaining these values that are less than 3%, a new study was carried out with the data that had already been obtained, in order to know if there is a significant difference between them, and for this an analysis of variance ANOVA was used (Table 4) [12, 13].

According to the results obtained in the analysis of variance, there is a significant difference; therefore we proceeded to make a comparison of means with Dunnett's test. In which Y1 was bought with Y2 and Y3 with Y2, using a confidence level of $\alpha = 0.05$, obtaining a D of teorical value of 0.3688 and the results obtained in the comparison of means were: 0.01α 0.3688 and 1.32α 0.3688 respectively. And in this way it was found that there is no significant difference between low and medium values [13].

Finally, the results obtained in the parameters evaluated in the analytical methods were compared with the results found in traditional methods and met

Table 2 ANOVA table for intermediate precision.

FV	gl	SC	MC	*F* cal	*F* tab
α_i	1	0.4764	0.4764	14.7676	18.5128
$\beta_{(i)}$	2	0.0645	0.0323	2.0399	4.4590
$\varepsilon\kappa_{(ij)}$	8	0.1265	0.0158		
Total	11	0.6675			

Table 3 ANOVA table for reproducibility.

FV	gl	SC	MC	*F* cal	*F* tab
α_i	2	0.1344	0.0672	0.1541	5.1433
$\varepsilon_{j(i)}$	6	2.6177	0.4363		
Total	8	2.7521			

Table 4 ANOVA table for robustness.

FV	gl	SC	MC	*F* cal	*F* tab
α_i	2	0.3820	0.1910	11.4821	5.1433
$\varepsilon_{j(i)}$	6	0.0998	0.0166		
Total	8	0.4818			

Table 5 Results in the parameters evaluated in the validation of the fat analysis method with hydrolysis.

Sample (matrix)	Method	Acceptance criteria	Results
Finished food for animal consumption (precision)	Primary or traditional	CV ≤ 2%	$\bar{Y}$ = 7.97 S = 0.04 CV = 0.52
	Semiautomatic equipment	CV ≤ 2%	$\bar{Y}$ = 7.21 S = 0.06 CV = 0.92
Finished food for animal consumption (intermediate precision)	Primary or traditional	CV ≤ 3%	Analyst A: CV = 2.26 CV =1.03 Analyst B: CV =2.74 CV =1.48
	Semiautomatic equipment	CV ≤ 3%	Analyst A: CV = 1.97 CV =1.62 Analyst B: CV =1.62 CV =1.82
Finished food for animal consumption (repeatability)	Primary or traditional	CV ≤ 2%	CV low = 2.09 CV medium = 1.48 CV high = 1.03
	Semiautomatic equipment	CV ≤ 2%	CV low = 15.86 CV medium = 1.97 CV high = 1.59
Finished food for animal consumption (robustness)	Primary or traditional	CV ≤ 3%	CV low = 1.60 CV medium = 1.52 CV high = 2.60
	Semiautomatic equipment	CV ≤ 3%	CV low = 2.09 CV medium = 1.97 CV high = 1.69

the parameters of reproducibility, repeatability, linearity, accuracy, precision, intermediate precision.

Table 5 shows the results that were obtained during the validation in the parameters described above (precision, intermediate precision, reproducibility and robustness), as well as the matrices that were occupied [13]. This table describes if the results obtained in the analysis are within the acceptance criteria, this indicates whether the result will be reliable for the future comparison between the traditional method and the method using semiautomatic equipment.

4. Discussion

In this study, a comparison of the fat analysis method with hydrolysis and the traditional method, which is widely described in the literature, was made against the method using semi automatized equipment; in order to demonstrate that using semi-automated equipment reliable results will be obtained with great advantages over the traditional method [12, 13]. To achieve this comparison, it was established through the evaluation of four parameters, that the performance characteristics of both methods satisfy the requirements for their analytical application [1].

As the first parameter, the precision was evaluated, which measures the degree of agreement between the analytical results obtained in several repeated analyze of the same analyte carried out under the same conditions. In the results of the study it was found that the method complies with the precision parameter, because the result is within the acceptance criteria, with a CV less than 2% [11, 13].

The CV was 0.52 in the results for the traditional method, compared to the one obtained when we used semi automatized equipment (0.92) which is smaller, however, the difference between both is not imply a variation that affects the results in a significant way, because both coefficients of variation are within the

acceptance criteria [13].

The intermediate precision allows expressing variations between different analysts, different days, different environmental conditions, different equipment or reagents, etc. In this study it was found that there is no variation between analysts, days or between analyst and day. The results obtained are within the acceptance criteria, the CV in all determinations is less than 3%. In a complementary way, a statistical analysis (ANOVA) was carried out, in which it is possible to determine if there is variation in the results when the method is carried out by different analysts or on different days. In this statistical analysis we have that the calculated *F* is less than the *F* of theoretical value, this indicates that there is no variation in the results, therefore, the method can be performed by different analysts or on different days and there will be a statistically acceptable variation [11, 13].

The difference between the coefficients of variation of the traditional method and the method with semiautomatic equipment is minimal, and both are within the acceptance criteria, using any of the methods will give us reliable results. The difference is presented in the advantages that the method with semi-automatic equipment gives us.

Reproducibility is the degree of agreement between the results of successive measurements of the same analyte using the same method under different conditions. There were 9 analyzes with 3 different weights: high, medium and high. In the medium and high levels, a CV was obtained within the acceptance criteria, which could be used without significant variation in the results. In addition, a statistical analysis of variance (ANOVA) was performed to determine if there was a significant difference in the results between the three levels of concentration, resulting in no variation between the three levels of concentration, since *F* calculated is less than *F* found in Fisher's tables [11, 13].

Regarding the robustness of an analytical method, it measures the ability not to be affected by small deliberate variations of the parameters of the method, 3 different concentrations of a hydrochloric acid solution were evaluated. In all the results the CV was less than 3%, being within the acceptance criteria. A statistical analysis (ANOVA) was carried out where it was found that there is a significant difference using different concentrations of the acid solution. Therefore, we proceeded to perform a statistical method to know which of these concentrations statistically with different results from those used in the concentration had indicated in the method; In the results of the Dunnett's test we have that there is no difference between low or medium concentrations of the acid solution [11-13].

Finally, and according to the results obtained in the parameters that were evaluated, the adequate conditions for carrying out the method are either of the two weights with CV less than 3%, because there is no significant difference between both weights. The concentration at which the acid solution must be found must be either of the two lower concentrations of low concentration. Under these conditions there will be no significant difference, when the method is executed by different analysts in different days.

The use of semi-automated equipment results in reduced analysis time, lower costs, savings in reagents and several tests can be developed simultaneously with the reliability of the results.

5. Conclusions

The semiautomated equipment allows optimizing the proximal analytic method, in comparison with the traditional official methods. Semi-automated equipment has enormous technical and economic advantages: from the technical point of view, less sample quantities, sample handling, less analysis of a larger number of samples, reduction of pollutant emissions, reduction of waste, less contamination, greater efficiency in analysis times, less human

resources than involved in the process, small sample retention spaces.

Regarding the reliability and traceability of the analytical data, using documented evidence of accuracy, precision, linearity, limit of detection and quantification, using semiautomated equipment does not have in this sense significant difference with traditional methods, which means that, using methods semi-automated has safety and reliability.

Because of the traditional methodologies, proximal determination requires greater manipulation of the samples, their analysis time is longer, involves more resources, frequent collection of waste, larger volumes of waste, resulting in a higher cost per test done. The use of semi-automated equipment is economical since it optimizes these operations.

The advance in using semi-automated equipment in food analysis laboratories will undoubtedly contribute to efficiency and increase productivity in them, since they offer very favorable advantages from the scientific, technical and economic point of view, having used the results of the determination of fat in a food matrix via hydrolysis (complicated method and by the traditional way) and its comparison allowed validating the analytical method and giving the degree of confidence to the use of semiautomatized equipment, in addition to the comparison between them, which gives technical foundation and statistical. Finally, we can say that development and use of analytical methodologies with these technologies is undoubtedly beneficial for food companies, pharmaceutical companies and all those that require proximal analysis, due to the enormous advantages offered by semi-automated equipment.

References

[1] Federal Commission for the Protection against Sanitary Risks (COFEPRIS)—Commission of Analytical Control and Extension of Coverage (CCAYAC), Criteria for the Validation of Physicochemical Methods. Mexico: Secretary of Health, 2011.

[2] National College of Pharmaceutical Chemists Biologists of Mexico A.C. 2002. *Guide to Validation of Analytical Methods.* Mexico: National College of Pharmaceutical Chemists Biologists of Mexico A.C.

[3] Internacional Conference On Harmonisation of Technical Requirements for Registration of Pharmaceutical for Human Use, Validation of Analytical Procedures Q2 (R1), ICH Harmonised Tripartite Guideline, 1996.

[4] International Organization for Standardization. ISO 9000. 2005. Quality Management Systems: Fundamentals and Vocabulary. Central Secretariat Geneva.

[5] International Organization for Standardization. ISO 9001 2008. Quality Management Systems: Requirements. Central Secretariat Geneva.

[6] Interinstitutional Commission of Good Manufacturing Practices. 2004. Validation Guide for Analytical Methods: Equipment Cleaning. Mexico: Interinstitutional Commission on Good Manufacturing Practices.

[7] Interinstitutional Commission of Good Manufacturing Practices. 2006. Good Validation Practices. Mexico: Interinstitutional Commission on Good Manufacturing Practices.

[8] Official Mexican Standard NOM-059-SSA1-2015. 2015. Good Manufacturing Practices for Medicines, Secretary of Health, Mexico.

[9] ICH-FDA. Guideline for Industry Text on Validation of Analytical Procedures; Notice. Federal Register on March 1, 1995, Vol. 60.

[10] ICH-FDA. Validación de métodos analíticos; notice. Federal Register on May 19, 1997, Vol. 62, No. 96.

[11] 1990. Official Methods of Analysis A.O.A.C. (15th ed.). U.S.A.

[12] NMX-F-427-1982. Foods. Determination of Fat (Acid Hydrolysis Method). Mexican Standards. General Direction of Standards.

[13] Cervantes M., et al. 2010. *General Aspects of Process Validation*. Mexico: UNAM FES Zaragoza.