ANOVA: A TOOL FOR BETTER EXPERIMENTAL DESIGN AND EVALUATION OF METHOD VALIDATION

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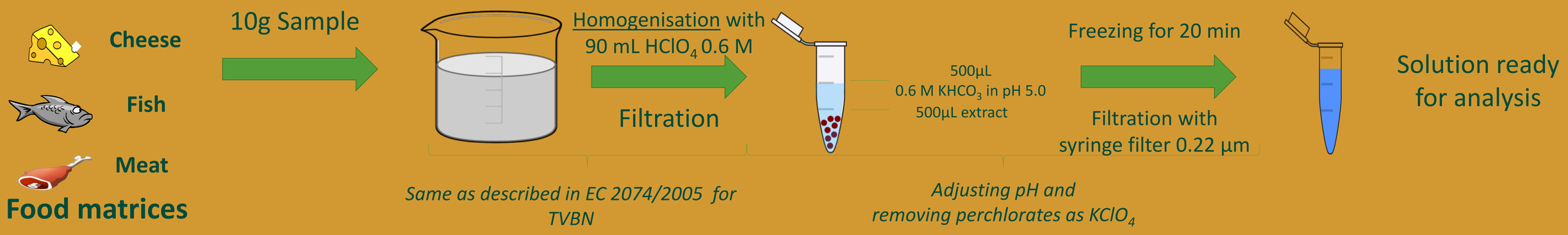
Abstract

Analysis of Variance (ANOVA) is a powerful tool for the estimation and separation of the different causes of variation. In the new edition of EURACHEM Guide "The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics", ANOVA is proposed as an alternative way for the simultaneous determination of intermediate precision and repeatability in a validation study. Nested ANOVA have been also proposed in literature, as another way for the estimation of trueness and uncertainty [1-3]. Moreover, through nested (hierarchical) experimental design, the source of variation (matrix, concentration or replicate) can be estimated and it helps the analyst to have a better view of methods' characteristics. The aim of this work is to explore the capabilities of ANOVA in validation. Through an example of method validation for biogenic amines in food matrices, the estimation of precision, trueness and uncertainty will be described with the same nested experimental design and the advantages and disadvantages for using ANOVA instead of classical approach will be discussed.

Sample pretreatment



Instrumentation & Method

LC-ESI-MS/MS
Thermo Scientific UHPLC Accela Pump-TSQ Access
ESI: **Positive**, MS Mode: **SRM**
Column: Luna HILIC Phenomenex
Mobile Phase (gradient elution):
A: Amm. Form. 50 mM, pH 4.00, B: ACN
Solvent dilution (ACN): 80:20

Statistical Software:
Minitab 16 v. 16.1.1, Microsoft Excel

Analyte: Biogenic Amines, example of Histamine

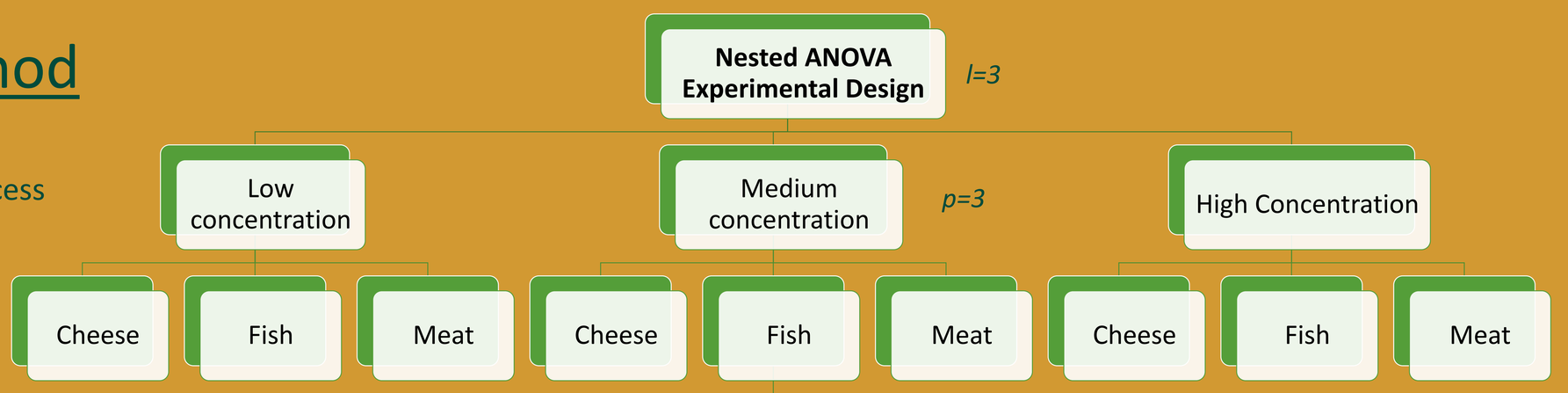
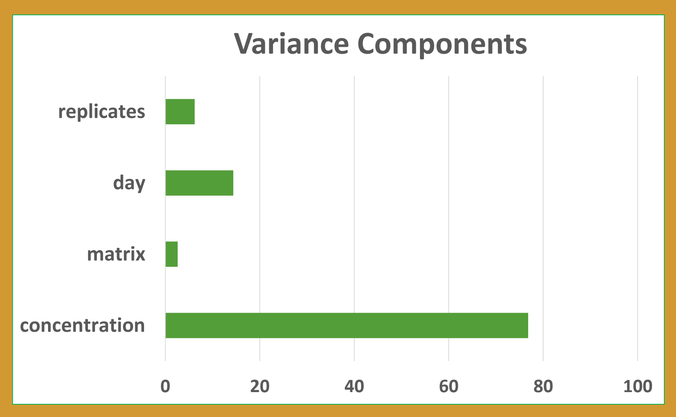


Table 1. ANOVA table as exported for statistical software for the validation of Histamine

Source	DF	SS	MS	F	P
Concentration	2	0.3410	0.1705	33.985	0.001
Matrix	6	0.0301	0.0050	1.599	0.186
Day	27	0.0847	0.0031	5.639	0.000
Error	36	0.0200	0.0006		



Plot 1. Percentage of Variance of every component of validation for histamine as calculated of statistical software

Precision (Intermediate precision + repeatability)

%RSD	ANOVA Approach	Classical Approach
Low Concentration	4.30	4.97
Medium Concentration	5.43	5.94
High Concentration	2.83	2.78

Better estimation because the calculation includes all components of variance and depends on the experimental design components. In low and medium concentration levels, there is larger variance of component of matrix that influences the classical approach and increases RSD.

Trueness

Overall recovery, $\overline{\overline{R_m}}$: 96.3%
Trueness evaluated through the calculation of overall uncertainty that includes components of intermediate precision, matrix and different concentration level. The recovery should not be statistical different than 100%. For large number of measurements and 95% confidence interval, t critical value is 1.96 of the normal distribution.
$t_{exp}=0.43 < t_{critical}=1.96$, there is no statistical significant bias, for 95% confidence interval.

Measurement of Uncertainty

Calculation of every component that included in uncertainty without any sophisticated calculation or experimental design

Uncertainty component (%)	Low concentration	Medium concentration	High concentration
Repeatability	2.83	2.45	1.73
Intermediate Precision	4.97	5.94	2.78
Recovery, matrix	1.54		
Recovery, concentration	8.30		

Conclusion

- **ANOVA is already known that it is a powerful statistical tool.**
- **Nested design simplifies the validation protocol that many times confuses the analysts.**
- **At the same time with one experimental design three main analytical characteristics, trueness, precision and uncertainty can be estimated.**
- **It is helpful due to the simplified calculations**
- **A disadvantage, the large number of runs and the large number of data required, makes the approach impractical in some cases.**

References

1. V.I. Boti, V.A. Sakkas, T.A. Albanis, Measurement uncertainty arising from trueness of the analysis of two endocrine disruptors and their metabolites in environmental samples Part I: Ultrasonic extraction from diverse sediment matrices, Journal of Chromatography A, 114,2007, 139-147.
2. P. Dehouck, E. Van Looy, E. Haghedooren, K. Deckers, Y. Vander Heyden, E. Adams, E. Roets, J. Hoogmartens, Analysis of erythromycin and benzoylperoxide in topical gels by liquid chromatography, Journal of Chromatography B, 794, 2003, 293-302.
3. D. A. Durden, Evaluation of the order of hierarchical structures for the calculation of method uncertainty using nested and other designs, Journal of AOAC International, 94, 2011, 1643-1649.