OVERVIEW PAPER

Design and Analysis of Experiments for Post Harvest Management and Value Addition Studies with Applications to Essential Oils

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ABSTRACT

Appropriately designed experiments are the key for discovering the effect of factors on responses measured at the different stages of the Post Harvest Management and Value Addition (PHMVA) value chain. There are several types of statistical methods that can be used to meet specific objectives of the study that match the specific experimental design used to produce the data. In this paper, the most commonly used experimental designs and types of analyses are highlighted using examples from studies conducted on essential oil content and composition from several aromatic plants grown in Mississippi and Wyoming, USA; and Bulgaria. The examples illustrate the importance of designed experiments and appropriate type of analyses to unlock the effect of agronomic (pre-harvest), processing (post-harvest) and post-processing (re-utilization of waste) factors on essential oil content and composition. The statistical methods used in these studies include Analysis of Variance (ANOVA), Repeated Measures Analysis, Linear Regression, Nonlinear Regression, and Multivariate Analysis. The aromatic plants considered include Peppermint, Spearmint, Japanese cornmint, Lavender, Hyssop, Junipers, and Rose.

Keywords: *aromatic plants, ANOVA, nonlinear regression, repeated measures, multivariate analysis*

INTRODUCTION

Experiments in a form of a single test or a series of tests are conducted in virtually all fields to discover how a process or a system is affected by different factors. This discovery usually leads to determining the best combinations of the factors to optimize the outputs. To have a better understanding of the process or the system, it is very important to identify the factors that can be controlled and those that cannot be controlled, and view them as depicted in Figure 1. A system may have several controllable factors, but in a given experiment, the available resources may not allow to consider all, but only the most important factors as controlled factors and the others will be considered as uncontrolled factors that are collectively represented by the error term (ε) in the Analysis of Variance (ANOVA) model or Regression model (Montgomery, 2009).

There are several experimental designs that match the objectives of the experiment, the number of controlled factors, and the layout of the experiment. These include, the Completely Randomized Design (CRD) – that allows comparison of the means of different treatments (or levels of a single factor of interest), the Randomized Blocks Design (RBD) – appropriate for a single factor of interest and one blocking factor, Factorial design - the most important class of designs that allow the study of two or more factors of interest concurrently. For each design, if data are collected repeatedly on each experimental unit, then Repeated Measures Analysis that uses a specific Covariance Structure is completed (Little et al., 1998). To get the most out of the resources spent on experiments, researchers often collect data on several response variables, and analyze each response variable separately. However, it is also possible to analyze several response variables together to compare means by doing Multivariate Analysis of Variance (MANOVA), or to reduce the dimension of the dataset by analyzing a few linear combinations of the variables (Principal Component Analysis), or by going through a more elaborate process to see whether the data are consistent with a prescribed structure (Factor Analysis), or to identify and quantify the association between two sets of variables (Canonical Correlation analysis), or to separate distinct sets of observations and allocating new observations to previously defined groups (Discriminant Analysis), or to form groupings based on similarities (Cluster Analysis) (Johnson and Wichern, 2007). Of these multivariate data analyses, a Cluster Analysis example is given in this paper (Zheljazkov and Astatkie, 2011).

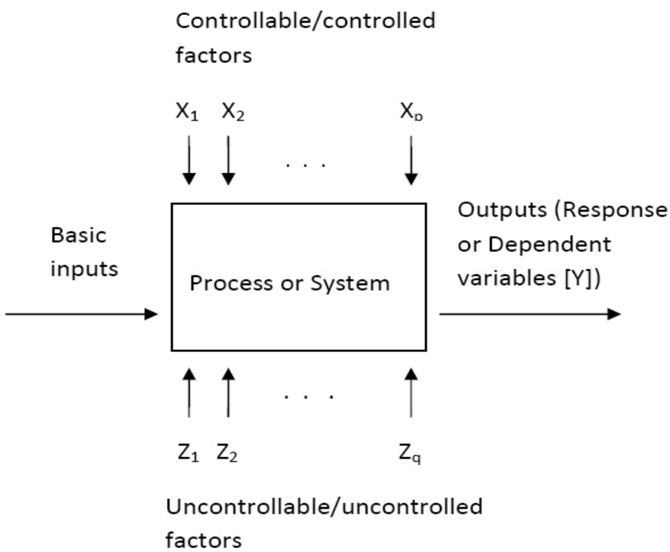

Figure 1. A general model that shows how a process or a system is affected by controllable and uncontrollable factors.

Data collected from such designed experiments can also be analyzed to identify the type of relationship between numerical factors and response variables, and then develop either linear or nonlinear regression models. Such models are used to describe the relationship and to predict values of the response variable at specified values of the numerical factors (Bates and Watts, 2007). For numerical factors, a blend of ANOVA and Regression methods are used to do Response Surface Analysis to explore the response surface and determine the optimum settings of the factors to either maximize or minimize the response (Myers *et al*. 2009).

PHMVA PHASES

Different sets of controllable factors (Figure 1) affect the different phases of the Post Harvest Management and Value Addition (PHMVA) value chain. For example, the essential oil content extracted from different aromatic plants are affected by agronomic and production factors in the first phase. To determine the effect of these factors, field or greenhouse experiments are conducted. In the second phase, another set of factors (processing and value addition factors) affect what happens post-harvest. The effect of these factors is determined by conducting laboratory experiments. In the third phase (post-processing), by-product reutilization and waste management factors are studied by doing either laboratory or greenhouse or field experiments.

STATISTICAL METHODS

Analysis of variance (ANOVA)

ANOVA is probably the most useful technique in the field of statistical inference (Montgomery, 2009). It is a method of comparing the means of different treatments or treatment combinations by partitioning the total variability in the response into the different components of the model, which matches the design of the experiment. For example, if there are two controllable factors (with a and b levels) affecting the system, and the a*b treatment combinations of these factors are formed by combining the levels of the factors, and if these treatment combinations are randomly assigned to the experimental units, then the design becomes an a X b factorial design. The model is:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ijk} \qquad (1)$$

where y_{ijk} is the response measurement from the ith level of the first factor, the jth level of the second factor, and the kth replication; μ is the overall mean of the response variable; α_i is the effect of the ith level of the first factor; β_j is the effect of the jth level of the second factor; $\alpha\beta_{ij}$ is the interaction effect of the two factors; ε_{ijk} is the error term representing the effect of all uncontrolled/uncontrollable factors (Figure 1), and is assumed to have normal distribution with constant variance, and independent of one another. The same assumptions are also used in linear and nonlinear regression models shown in Eq. 2 to Eq. 8. The subscript *i* represents the levels of the first factor and goes from 1 to a, *j* represents the levels of the second factor and goes from 1 to b, and *k* represents the replications and goes from 1 to n. Once the validity of these assumptions is verified by examining the residuals (Montgomery, 2009), the p-values from the ANOVA table are looked at to determine the significance of each effect, and if significant the next step is to do multiple means comparison to identify the treatment or treatment combination that gives the most desired result.

Regression

Regression analysis is a powerful tool of uncovering the type of relationships between an independent (or a numerical factor) and a dependent variable (or a response variable). While almost all relationships are nonlinear, most of them can be adequately approximated by linear models. As Bertrand Russell said, "Although this may seem a paradox, all exact science is dominated by the idea of approximation" (Bates and Watts, 2007). A regression model is linear if all its derivatives with respect to each parameter do not involve another parameter. On the other hand, a regression model is nonlinear if at least one of the derivatives of the model with respect to each parameter involves at least one parameter. That is, for a relationship to be linear, it does not have to be straight line. For example, second and third order polynomial models are not straight line, but they are linear models.

Linear regression models are either Simple Linear Regression model if there is only one independent variable; or Multiple Linear Regression model if there are two or more independent variables. For example, a Multiple Linear Regression model with two independent variables x_1 and x_2 is written as:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_x + \varepsilon \qquad (2)$$

Nonlinear regression models are usually developed for one independent variable, and the two main differences between linear and nonlinear models are: 1) nonlinear models are usually based on the inherent mechanistic relationship between the variables, and as a result they describe the relationship better, and are more reliable to use them for prediction purposes than linear models; 2) while estimating the parameters of linear regression models is easy, the estimation of nonlinear regression parameters is not, mainly because the parameters are estimated iteratively, as a result of which convergence and estimability challenges may arise (Bates and Watts, 2007). For example, the three-parameter Asymptotic Regression model is nonlinear:

$$y = \theta_1 - \theta_2(\exp(-\theta_3 x) + \varepsilon \qquad (3)$$

The first step in regression analysis is plotting x vs. y to learn more about the form and strength of the relationship. Then the parameters of linear models can be estimated using Proc REG of SAS, and the parameters of nonlinear regression models are estimated using Proc NLIN of SAS (SAS, 2010).

APPLICATIONS TO ESSENTIAL OIL STUDIES

1 Pre-harvest phase

Three examples from studies conducted to determine the effect of agronomic and production factors on essential oil content are given below.

1.1 Study on Japanese cornmint in Mississippi (Zheljazkov *et al.,* 2010): Japanese cornmint (also known as Mentha Arvensis; Figure 2) is grown for production of essential oil, from which (-)-menthol is extracted. Both (-)-menthol and the de-mentholized oil are widely used as a flavor and fragrance vectors in the pharmaceutical, food, flavor, and fragrance industries. In spite of its importance and even if the USA is a major market for Japanese cornmint essential oil, there is no commercial production of it in the USA (Zheljazkov *et al.*, 2010).

The objective of this field experiment was to determine the effect of Cultivar (Arvensis II and Arvensis III) and N rate (0, 80, 160 kg/ha) on Japanese cornmint essential oil yield and the composition of several constituents. The responses were measured repeatedly on two occasions (cut 1 and cut 2), and the experiment was done over two years. The experimental design was a 2 X 3 factorial, and the response measurements were analyzed as Repeated Measures with Cut nested in Year.

The main findings of this study were: 1) demonstration that Japanese cornmint could be grown successfully in Mississippi and possibly in other southeastern USA; and 2) discovering interaction effects of the three factors, which allow the determination of preferred specific treatment combinations for a specific essential oil constituent; 3) the finding that essential oil, menthol, and menthone yields were higher from the first cut.

Figure 2. Leaves, stems, and inflorescences of the two Japanese cornmint cultivars "Arvensis II" and "Arvensis III" used in the study

1.2 Lavender and hyssop productivity, oil content, and bioactivity as a function of harvest time and drying (Zheljazkov *et al.*, 2012): Lavender and hyssop (Figure 3) are widely grown essential oil crops in Europe. Their essential oils are used in perfumery and cosmetics, eco-friendly pesticides, foods, beverages, liqueurs, pharmaceuticals, and traditional medicines in Asia, Europe, ancient Greece and Room. Lavender and hyssop were mentioned in the bible, and in ancient Jewish texts (Tonutti and Liddle, 2010). The objective of this field experiment was to determine the effect of Material (fresh, dry) on essential oil yield and the composition of several constituents.

Figure 3. Lavender (*Lavandula angustifolia Mill.*) – left, and Hyssop (*Hyssopus officinalis L.*) – right used in the study

The response variables were measured repeatedly on three occasions (Harvest: 1, 2, 3), and the experiment was done over two years. The design of the experiment was Randomized blocks design (RBD), and the data were analyzed as Repeated Measures with Harvest nested in Year because the plant in the second had a different canopy structure that might affect the response measurements. This was the first study that demonstrated Lavender and Hyssop could be grown successfully in southeastern USA. The results also indicated that the essential oil content and compositions obtained were similar to those from traditionally growing countries in Europe, the Middle East, Asia, and Northern Africa. The agronomic factors considered in the experiment, namely Material and Harvest, can also be customized to maximize the major constituents of Lavender (linalool and linalylacetate) and Hyssop (pinocamphene and β-pinene).

1.3 Yield, content and composition of peppermint and spearmints as a function of harvesting time and drying (Zheljazkov *et al.*, 2010): Peppermint and Spearmint are extensively used as aromatic agents in chewing gum, toothpaste, mouthwashes, and in pharmaceuticals, confectionary, aromatherapy, as antimicrobial agents, and in eco-friendly pesticides. They are widely grown in northern latitudes, but there is very limited info on how agronomic and production factors affect them south of the 41st parallel. Therefore, this experiment was conducted to determine the effect of Cultivar (B90-9, Black Mitcham for Peppermint; Native, Scotch for Spearmint) and Material (fresh and dry) on essential oil content and several constituents, as well as antioxidant capacity.

The experimental design for each plant (peppermint and spearmint) was a 2 X 2 factorial design, but since the response variables were measured repeatedly on 6 harvest dates, Repeated Measures Analysis was conducted for the ANOVA, and three regression models were used to describe the relationship between harvest time (days since transplantation) and the response variables. The relationships were adequately described either by a 3-parameter Gompertz regression model (Eq. 4) or a 4-parameter Logistic regression model (Eq. 5), both of which are nonlinear, or a third order polynomial (Eq. 6), which is linear:

$$y = \theta_1 e^{-e^{-\left(\frac{x-\theta_2}{\theta_3}\right)}} + \varepsilon \quad (4)$$

$$y = \theta_0 + \frac{\theta_1}{1+\left(\frac{x}{\theta_2}\right)^{\theta_3}} + \varepsilon \quad (5)$$

$$y = \beta_0 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \varepsilon \quad (6)$$

Figure 5 shows plots of *x* vs. *y* together with the fitted 4-paramter Logistic regression model for six responses. This figure demonstrates how well the relationships were described by this nonlinear regression model. Highlights of the main findings include 1) peppermint oil from dried material had higher menthol and eucalyptol concentration, 2) Menthone in peppermint was higher in early harvests, than later, 3) Carvone in spearmint accumulated early, allowing early harvest, and 4) Spearmint and peppermint essential oil have high antioxidant activity.

Furthermore, this was the first comprehensive study on how harvesting and post-harvest management would affect the essential oil composition of commercial varieties of peppermint and spearmint. This was also the first study to compare the antioxidant, antimicrobial, antileishmanial, and antimalarial activities of oils from these mint species produced in the hot humid conditions of the southeastern USA.

2 Post-harvest phase

Three examples from studies conducted to determine the effect of processing and value addition factors on essential oil content are given below.

2.1 Improvement of essential oil yield of oil-bearing (*Rosa damascena* Mill.) due to surfactant and maceration (Dobreva *et al.*, 2011): Bulgaria produces the most essential oil from rose, and the highest quality rose essential oil is produced from the Bulgarian Kazanluk rose, which is a form of Rosa damascene. But, since the essential oil content from the Kazanluk rose and other oil bearing roses is only around 0.3 to 0.4 $ml.kg^{-1}$ in fresh flowers (Kovatcheva *et al.*, 2011), there is a strong need to find ways of increasing yield. With this in mind, the study was conducted in Bulgaria to determine the effect of Tween 20 (polyoxyethylene sorbitan monolaurate) with and without maceration, and flowering stage (beginning, full bloom, and end) on essential oil content and constituents.

The experimental design was a 3 X 5 factorial design, with the two factors being Phenological phase (beginning, full, end) and Treatment (control, T [Tween] at 1000, T at 1000 + M, T at 2500, T at 2500 + M). ANOVA followed by multiple means comparison results (Figure 4) showed that Tween 20 at 2500 mg L^{-1} rate plus Maceration of the flowers immediately after cutting and prior to distillation increases essential oil yield by 94%. This finding will offer commercial rose producers in Bulgaria a practical tool for increasing essential oil yields and improving economic and environmental sustainability of rose essential oil production.

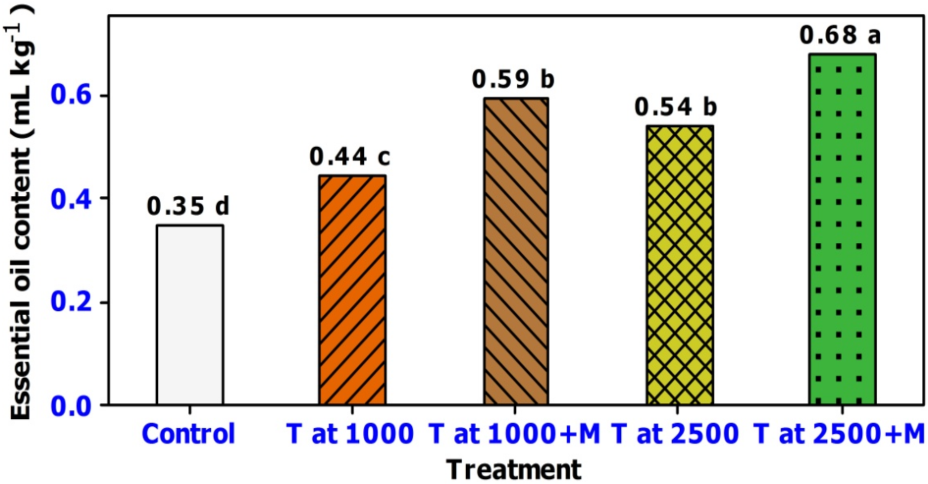

Figure 4. Mean essential oil content from the five treatments. Means sharing the same letter are not significantly different at the 5% level of significance.

2.2 Effect of distillation time on Mentha canadensis essential oil yield and composition (Zheljazkov and Astatkie, 2012): Mentha canadensis, which is synonymous of Mentha Arvensis and Japanese cornmint is a subtropical plant grown widely in India, Vietnam, China, in some south American countries (Brazil), and in a smaller acreage in eastern Europe (Bulgaria, Romania) (Chand *et al.*, 2004). Various researchers used different distillation time (DT) for extraction of Mentha canadensis, ranging from 60 minutes to 180 minutes, however, it is now known how essential oil and composition are affected by DT. This lab experiment was conducted to determine the effect of distillation time (DT: (1.25, 2.5, 5, 10, 20, 40, 80, and 160 min) on essential oil content and composition, and develop regression models to describe the relationship between DT and essential oil content and composition.

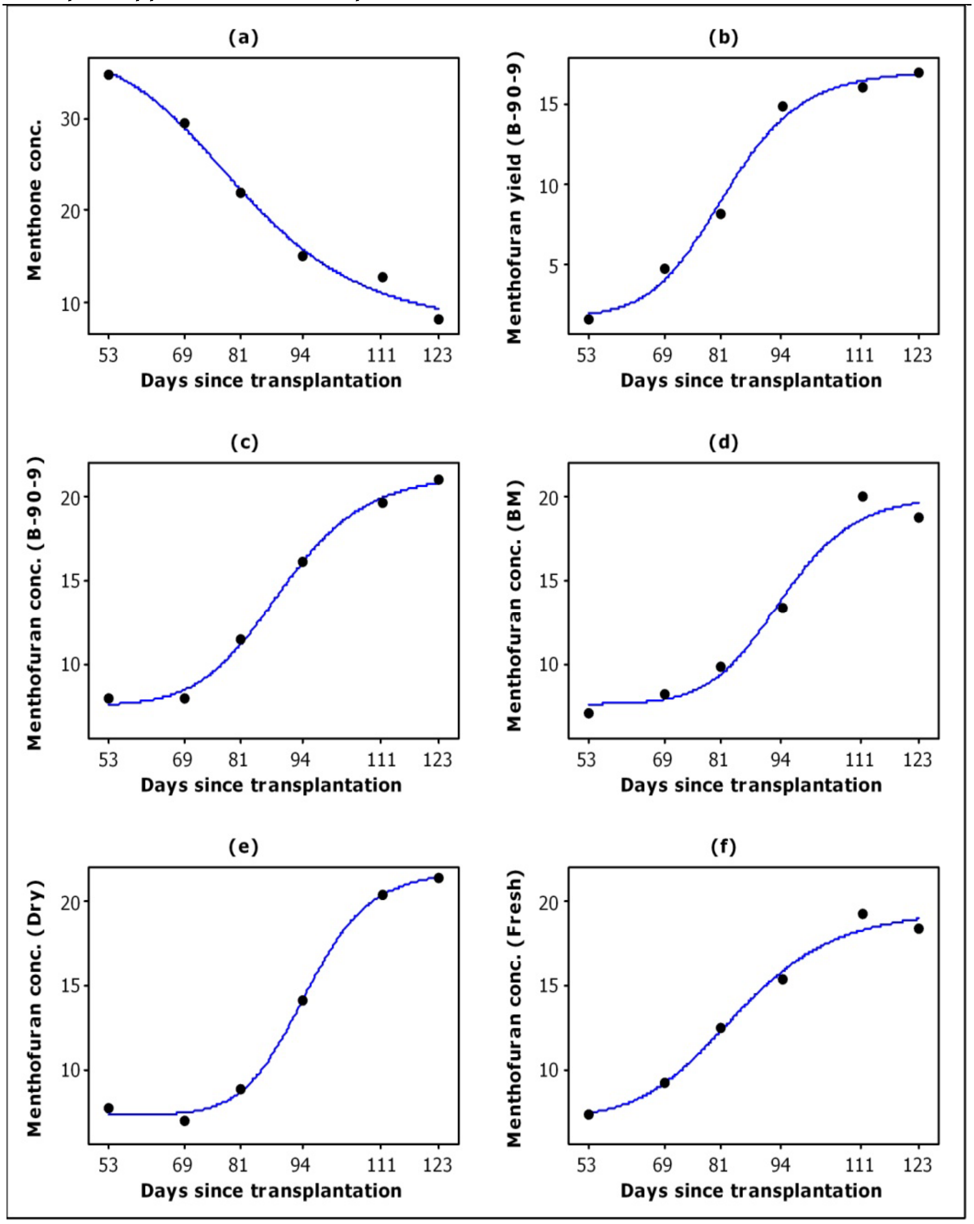

Figure 5. Fitted 4-parameter logistic regression model of (a) menthone concentration (%), (b) menthofuran yield (kg ha^{-1}), and (c-f) menthofuran concentration (%) on the number of days since transplantation for the two materials and the two cultivars of peppermint.

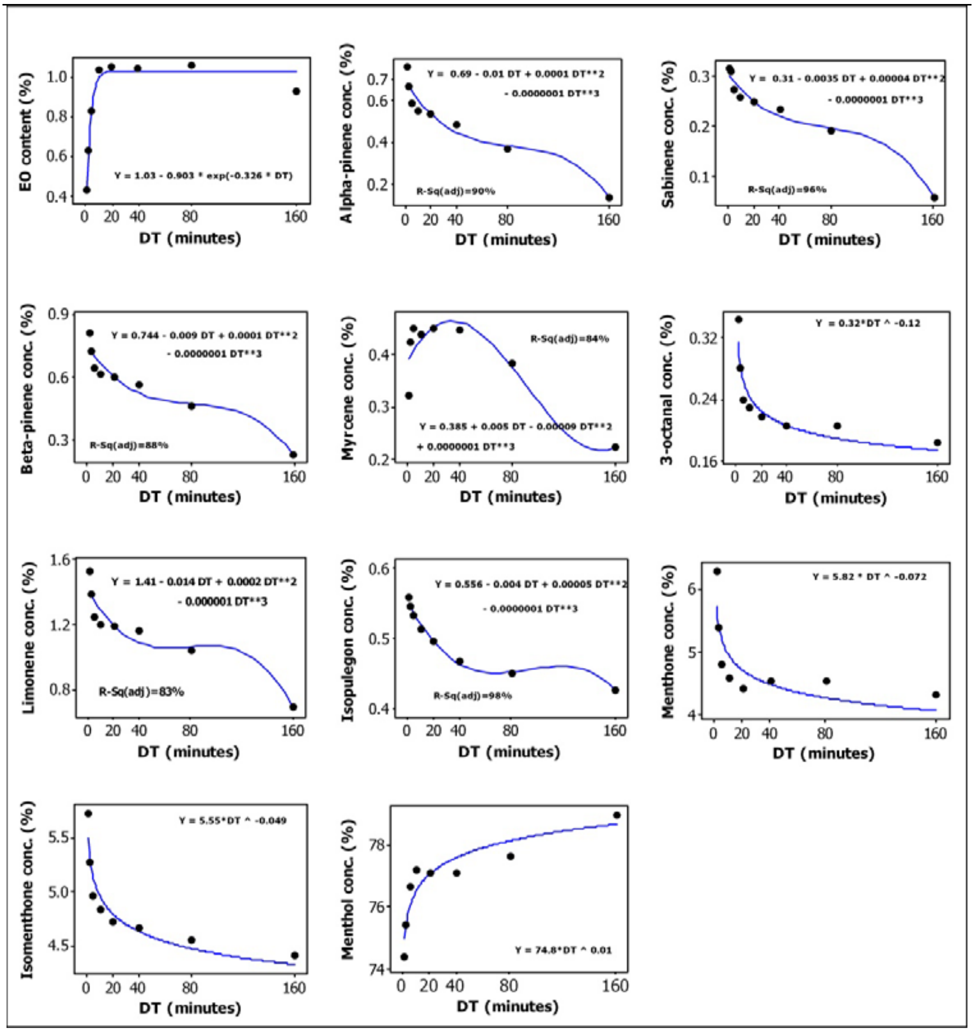

Figure 6. Plots of essential oil (EO) content and the concentrations of 10 compositions vs. distillation time (DT) together with the fitted Asymptotic, Power and third-order polynomial regression models. Equations of the fitted models are shown within each plot.

ANOVA was completed using the Completely Randomized design (CRD), and the regression models used to describe the relationships were 1) the Asymptotic model (Eq. 3), 2) a third-order polynomial (Eq. 6), and 3) the Power model (Eq. 7), which is nonlinear.

$$y = \theta_1 x^{-\theta_2} + \varepsilon \qquad (7)$$

The main findings of this study were: 1) essential oil reached maximum at 10 min DT, which is much lower than the Industry standard of 60 minutes or more; 2) as can be seen in Figure 6, the relationship between DT and the compositions were not the same, which suggests that DT can be customized to optimize the yields of specific compositions; 3) this work can be used as a reference when comparing reports that used different DT.

2.3 Distillation time alters essential oil yield, composition, and antioxidant activity of male *Juniperus scopulorum* trees (Zheljazkov *et al.*, 2012): Juniperus has several species spread all over the world. Rocky Mountain juniper (*Juniperus scopulorum* Sarg.) is one of the most widespread Junipers in the Western US, Canada, and Mexico. Rocky Mountain juniper contains two natural products; essential oil and podophyllotoxin, and has been used as medicinal plant by native Indians for treatment of various diseases (Lind, 2005). Rocky Mountain juniper has been a subject of numerous studies on how it is affected by different factors; however, there are no reports on the effect of the length of distillation time on essential oil composition and yield. Literature reports usually cite 90 min or 120 min DT for some Junipers (Adams, 2011). The objective of this study was to determine the effect of distillation time (DT: 1.25, 2.5, 89 5, 10, 20, 40, 80, 90 160, 240, 360, 480,600, 840 and 960 min) on essential oil content, composition and antioxidant activity, and develop regression models to describe their relationship using samples taken from Rocky Mountain Juniper male cones in Wyoming, USA.

The ANOVA was completed using the completely randomized design (CRD), and the relationships were adequately described by the 1) Power model (Eq. 7), and 2) the Michaelis-Menten (Eq. 8) nonlinear Regression models.

$$y = \frac{\theta_1 x}{\theta_2 + x} + \varepsilon \qquad (8)$$

The main findings include: 1) unlike the standard DT of 120 min, the highest essential oil yield was obtained at 840 min; 2) the concentration of the low-boiling constituents: alpha-thujene, alpha-pinene, sabinene and para-cymene were higher at the shortest DT; 3) the highest (50% more) antioxidant activity was achieved at 480 min DT.

3 Post-processing phase

In this sub-section, I offer one example to demonstrate the role of statistics in understanding the influence of re-utilization of waste factors on essential oil content.

3.1 Effect of distillation waste water and plant hormones on Spearmint growth and composition (Zheljazkov and Astatkie, 2011): Spearmint (*Mentha Spicata* L.) is a major essential oil crop in USA. For example, in 2008, 1.09 million Kg of Spearmint essential oils were produced in USA, which are used as major aromatic agents with wide application in the food and pharmaceutical industries and in a number of consumer products, including chewing gum, toothpaste, and mouthwash. Distillation waste water (DWW) is a waste product from the steam distillation of essential oil crops, which, currently is released into streams and rivers, causing environmental concern.

The objective of this study was to determine the effect of DWW from 13 essential oil crops, extracts from 2 alkaloid containing species, and 3 hormones on essential oil content and constituents of Spearmint, and investigate similarities among the treatments and the constituents.

In addition to ANOVA of a completely randomized design (CRD), multivariate analysis, particularly cluster analysis was used to determine similarities among the treatments (on how they affect the responses, Figure 7), and among the constituents (on how they are affected by the treatments, Figure 8).

The main findings of this study were 1) DWW of some essential oil crops can alter the concentration of individual constituents 'Native' Spearmint essential oil; and 2) the effect of DWW from some essential oil crops was similar to that of the plant hormones (MJ, GA3 and SA), which suggests that DWW can replace these plant hormones and save resources and the environment.

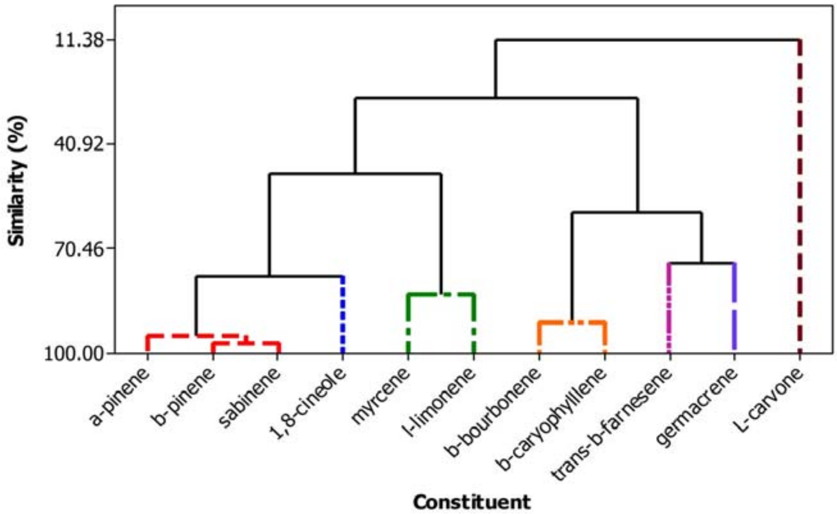

Figure 7. Complete linkage dendrogram of similarities among the 11 constituents. Constituents within the seven clusters had more than 83% similarity.

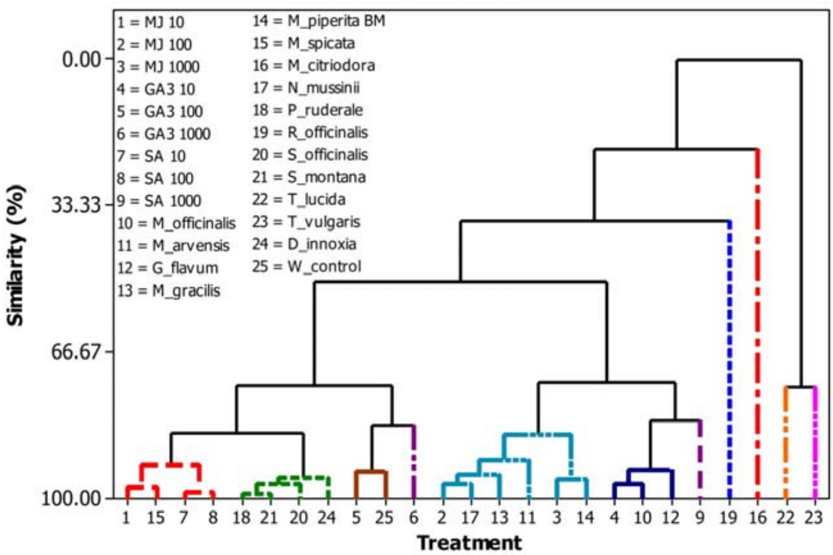

Figure 8. Complete linkage dendrogram of similarities among the 25 treatments. Treatments within the eleven clusters had more than 85% similarity.

CONCLUSIONS

This paper reviewed Analysis of Variance, Regression modelling and Multivariate data. Analysis, and showed applications of these statistical methods to learn about a process or a system. The knowledge gained would then allow the determination of the best and worst settings of factors to achieved desired outputs from the system. These factors could be either from one or combinations of pre-harvest (production), or post-harvest (processing), or post-processing (re-utilization of waste). The examples used in this paper came from our recent studies on essential oil and compositions that targeted all these three phases of the PHMVA value chain. Through these studies, among others, we were able 1) to demonstrate that Japanese cornmint, Lavender, Hyssop, Peppermint and Spearmint could be grown successfully in Mississippi and possibly in other southeastern USA; 2) to compare the antioxidant, antimicrobial, antileishmanial, and antimalarial activities of oils from commercial varieties of peppermint and spearmint produced in southeastern USA; 3) to discover that the using Tween 20 with maceration treatment almost doubles essential oil yield from Bulgarian Kazanluk rose; 4) to determine the optimum distillation time for different plants and different essential oil constituents; and 5) to determine how distillation waste water, currently being released to streams and rivers and is causing environmental damages, can be re-utilized for growing aromatic plants. These findings could benefit commercial producers of essential oils as well as researchers who continually conduct studies to discover new knowledge.

These examples demonstrated the power and applications of design and analysis of experiments. However, I would like to re-emphasize that the specific statistical method used should be in line with the way the data were produced, and the objectives of the study; and before using the results, the validity of all model assumptions should be verified.

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