Optimum conditions for testing *N*-nitrosamine formation potential (FP) through ozonation in wastewater samples

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Abstract

N-nitrosamines are potential carcinogens that are formed during disinfection by chlorination/chloramination or ozonation. In this study, the main objective was to optimize ozonation experimental conditions (pH and temperature) for the production of *N*-nitrosamines by using the CCD method in wastewater matrices. As the results, maximum NDMA FPO3 (formation potential by ozonation) occurred at pH 8.96 and a temperature of 20.0 °C. Furthermore, attempts to reduce the concentrations of eight *N*-nitrosamines revealed that none was removed or reduced in concentration by ozonation using Milli-Q water for 2 h.

Key words: N-nitrosamines, Formation Potential (FP), Ozonation, Wastewater Treatment Plant (WWTP), Response surface methodology (RSM), Central composite design (CCD)

1. Introduction

N-nitrosamines are not new contaminants—their potential carcinogenic effects have been studied for over 40 years. In 1976, for example, researchers proposed that increased levels of

N-nitrosodimethylamine (NDMA) in the air around industrialized urban centers could be responsible for increases in the rates of certain cancers [1]. Studies have revealed that such *N*-nitrosamines are formed by chloramination [2-7].

Most studies of NDMA and ozonation have dealt mainly with reduction of NDMA concentrations rather than with the formation of this contaminant as an oxidation byproduct. For example, the reaction rate constants of NDMA with ozone and with hydroxyl radical ($\text{HO}\cdot$) are $(5.2 \pm 0.16) \cdot 10^{-2}$ and $(4.5 \pm 0.21) \cdot 10^8 \text{ M}^{-1}\text{s}^{-1}$, respectively, and NDMA is predominantly decomposed by $\text{HO}\cdot$ during ozonation [8]. Lee *et al.* [9] reported that, when selected NDMA precursors were pre-ozonated, NDMA formation potential (FP) by chloramination was decreased. More recently, however, several studies of NDMA formation by ozonation have been reported. Andrzejewski *et al.* [10] reported that NDMA is formed from dimethylamine by ozonation. Another study [11] showed that a plant growth regulator, daminozide, and its decomposition product (1,1-dimethylhydrazine), as well as a fungicide, tolylfluanide, and its decomposition products (*N,N*-dimethyl-*N'*-p-tolylsulphamide and dimethylsulfamide), are NDMA precursors in ozonation.

Recently, NDMA was found in high concentrations ($>100 \text{ ng/L}$) in some German drinking waters after ozonation [11]. This was unexpected, because NDMA is typically formed only in the low-ng/L range as an ozonation byproduct [10,12-15]. The NDMA precursor was determined to be *N,N*-dimethylsulfamide (DMS), a previously unknown metabolite of the fungicide tolylfluanide [11]. A survey in Germany revealed DMS concentrations as high as $18 \mu\text{g/L}$ in groundwaters [11]. This metabolite is transformed into NDMA at high yield (29% to 52%) during ozonation of in nature, and NDMA concentrations of 2 to 310 ng/L were found post-ozonation. These observations led to suspension of the use of tolylfluanide in Germany and other European countries. However, studies aimed at optimizing the conditions for ozonation experiments to determine which conditions produce the most NDMA are lacking. Some of the studies that have been done on experimental ozonation conditions are listed in Table 1.

(Insert Table 1)

Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes [19]. The most extensive applications of RSM are in the particular situations where several input variables potentially influence some performance measure or quality characteristic of the process. Thus

the performance measure or quality characteristic is called the response. The input variables are sometimes called independent variables, and they are subject to the control of the scientist or engineer. The field of response surface methodology consists of the experimental strategy for exploring the space of the process or independent variables; empirical statistical modeling to develop an appropriate approximating relationship between the yield and the process variables; and optimization methods for finding the values of the process variables that produce desirable values of the response. RSM uses an experimental design such as the central composite design (CCD) to fit a model by the least-squares technique [20,21]. The adequacy of the proposed model is then revealed by using the diagnostic checking tests provided by analysis of variance (ANOVA). The response surface plots can be employed to study the surfaces and locate the optimum. In several industrial processes, RSM is almost routinely used to evaluate the results and efficiency of operations [22-26]. Hence, in this study, the main objective was to optimize ozonation experimental conditions (pH and temperature) for the production of *N*-nitrosamines by using the CCD method in wastewater matrices.

2. Materials and Methods

2.1. Chemicals

N-nitrosodimethylamine (NDMA), *N*-nitroso-*n*-methylethylamine (NMEA), *N*-nitrodiethylamine (NDEA), *N*-nitrosodi-*n*-propylamine (NDPA), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosopiperidine (NPIP), *N*-nitrosomorpholine (NMOR), and *N*-nitrosodi-*n*-butylamine (NDBA) were purchased from Supelco (Bellefonte, PA). Deuterated *N*-nitrosodimethylamine-*d*₆ (NDMA-*d*₆), *N*-nitrosodi-*n*-propylamine-*d*₁₄ (NDPA-*d*₁₄), and *N*-nitrosopyrrolidine-*d*₈ (NPYR-*d*₈) were used as internal standards, and toluene-*d*₈ was used as an injection internal standard; these chemicals were purchased from Cambridge Isotope Laboratories (Pointe-Claire, PQ, Canada). As the corresponding isotope-labeled analogs were not available for all of the compounds, we used NDPA-*d*₁₄ as an internal standard for quantification of NDBA and NPYR-*d*₈ for NMEA, NDEA, NPIP, and NMOR. Individual stock solutions of the standards were prepared at 1 mg/L in dichloromethane (DCM) and stored at low temperature (−20 °C). Working standard mixtures (10 to 250 µg/L) of the compounds were prepared daily and used in standard curve preparation. GC-MS-grade DCM

was obtained from Wako (Tokyo, Japan). Ultra pure water (Milli-Q) was obtained from Milli-Q-Plus (Millipore, USA).

(Insert Table 2)

2.2. Optimization of conditions for testing FP_{O_3} by ozonation using a CCD experiment

We defined the *N*-nitrosamines FP by ozonation as *N*-nitrosamines FP_{O_3} . Response surface methods are used to examine the relationship between one or more response variables and a set of quantitative experimental variables or factors. These methods are often employed after researchers have identified a few “vital” controllable factors and they want to find the factor settings that optimize the response. Designs of this type are usually chosen when researchers suspect curvature in the response surface.

2.3. Preparation of wastewater influent

To optimize the ozonation experiment, influent samples were collected from wastewater treatment plant (WWTP) N (Table 3), which was located in an industrial area of Japan. The characteristics of the influent at WWTP N are shown in Table 4. All influent samples were collected in 1-L amber glass bottles, kept in the dark at 4 °C, and analyzed within 10 h as grab samples. The pH of the test waters to be spiked with the *N*-nitrosamines was adjusted to 7, 9, or 11 with a phosphate buffer solution of 0.1 M prepared with NaH_2PO_4 , NaOH, and HCl solutions before each experiment. The temperature of the tested water was maintained at 10, 20, or 30 °C by using a water circulator to circulate hot water into a water jacket outside the reactor.

(Insert Table 3)

(Insert Table 4)

2.4. Ozone reactor system

All experiments were performed in a cylindrical stainless-steel reactor with an inside

diameter of 23 cm, a height of 40.7 cm and an effective volume of 1.5 L (Fig. 1). All the experiments started with the sparging of O₃ gas continuously into the reactor, which was filled with the influent.

(Insert Figure 1)

2.5. Reduction in concentrations of eight *N*-nitrosamines by ozonation

Removal of *N*-nitrosamine (especially NDMA) by ozonation has been reported previously [16]. In contrast, however, Fouillet *et al.* [27] found that NDBA and NMOR were not degraded by ozone [27]. Therefore, initially, to test whether *N*-nitrosamine concentrations could be reduced by ozonation, we performed several experiments using Milli-Q water spiked with eight *N*-nitrosamines. The *N*-nitrosamines were spiked into the Milli-Q water as 400-ng/L standard solutions; the ozone feed rate was 3.1 mg/L/min and the retention time was 120 min (Fig. 2). None of the eight *N*-nitrosamines was removed by ozonation.

(Insert Figure 2)

2.6. Pretreatment and analytical method

First, 200 mL of each corrected sample was filtered with a GF/B glass fiber filter. Then the deuterated forms of NDMA (NDMA-*d*₆; CDN Isotopes, Pointe-Claire, Canada), NDPA (NDPA-*d*₁₄; CDN Isotopes), and NPYR (NPYR-*d*₈; CDN Isotopes) were added to all filtrate samples as internal standards and the *N*-nitrosamines in the filtrate were concentrated in a Sep-Pak NH2 cartridge (as a sample clean-up step) and an AC-2 cartridge at a rate of 10 mL/min. Use of the NH2 cartridge (silica-based polar-bonded phase with basic character) substantially reduces the amount of co-extracted acidic interferences. The cartridges were conditioned in advance with 5 mL of DCM, 5 mL of methanol, and 5 mL of Milli-Q water. After concentration, only the AC-2 cartridge was dehydrated, using a pneumatic pump (Ulvac, DA-6-S, Osaka, Japan), for 2 h, in order to remove the remaining water in the cartridge. *N*-nitrosamines were eluted from the dehydrated cartridge with 2 mL of DCM. The eluate was further concentrated with a gentle stream of N₂ gas at 35 °C just before dryness. The residue was reconstituted in 200 µL of DCM and subjected to *N*-nitrosamine quantification by GC-MS/MS. To correct for fluctuations in the GC-MS/MS apparatus, an injection internal

standard (toluene-*d*₈: CDN Isotopes) was added just before injection of the samples into the system.

(Insert Figure 3)

Separation of the eight *N*-nitrosamines, their three internal standards, and the injection standard (toluene-*d*₈) was performed in less than 15 min. The chromatograms of the eight *N*-nitrosamines (250 µg/L spiked Milli-Q water extract) are shown in Figure 4.

(Insert Figure 4)

The eight *N*-nitrosamines, their three internal standards, and the injection internal standard were analyzed on a Varian 300 triple-quadrupole mass spectrometer (Varian, Tokyo, Japan) coupled to a Varian 450 gas chromatograph (Varian). Chromatographic separation was achieved in a FactorFour VF-17 ms capillary column (Varian; 30 m length, 0.25 mm ID, and 0.25 µm film thickness). Specific information details of GC-MS/MS are given in Table 5.

(Insert Table 5)

3. Results and discussion

3.1. Methodology and design of experiment

We used RSM to evaluate the individual and cumulative effects of the variables (namely pH and temperature) influencing the NDMA FP_{O3}. For this purpose we used a 13 full-factorial CCD, which is part of the RSM, and MINITAB® Release 14.20 software. The design therefore required 13 experimental values for the three-level factorial CCD. Experiments were performed accordingly whereby the pH was changed from 7 to 11 and the temperature from 10 to 30 °C (Table 6). Significant changes in NDMA FP_{O3}/NDMA FP_{O3maximum} were observed for all combinations, implying that both variables significantly affected the production of NDMA. The behavior of the system is explained by the following second-order quadratic equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{12} X_2^2 \quad (1)$$

where Y= Predicted response; X_1 and X_2 = main effects; β_0 = a constant; β_1 and β_2 = linear coefficients; and β_{11} and β_{12} = quadratic coefficients.

(Insert Table 6)

3.2. Analysis of technical statistics on actual survey data

Seven of the eight *N*-nitrosamines were not produced under the optimum conditions of pH 9 and a temperature of 20 °C. Only NDMA was produced under all of the study conditions. We therefore considered that the precursors of *N*-nitrosamines other than NDMA did not exist in the samples, or that conditions other than pH 9 and a temperature of 20 °C were not suitable for producing *N*-nitrosamines other than NDMA. Therefore, we focused only on NDMA for optimization of the conditions of FP testing by ozonation. During the batch test, ozonation was performed at several different reaction times, as used by Kosaka *et al.* [16]. Therefore, the reaction times in the main experiment were set at 10, 15, and 20 min and the values that gave the maximum NDMA FP_{O3} were chosen.

A response surface regression analysis was performed to determine the significance of the regression coefficient of each variable. Regression coefficients, standard errors of the coefficients, *T* values, and significance probability values (*P* values) (all estimated using uncoded values) are given in Table 7. The regression coefficient of the constant was 8.807, with a *T* value of 4.907 and a *P* value of 0.001. Thus the value of the constant was significant (high *T* value and low *P* value). Moreover, the value of the constant did not depend on either a linear term, a square term, or interaction terms of the variables. Table 7 also shows that the linear term “pH” was significant (*P* < 0.05), with a high *T* value (*T* = 4.596). However, the quadratic term of pH was also significant (*P* = 0.002), with *T* = 4.632; the *T* value in the case of the quadratic term was higher than in the case of the linear term of pH. This indicated that there was a curve relationship between pH and NDMA FP_{O3}/NDMA FP_{O3maximum}. Similarly, the linear term “temperature” was also significant, (*P* = 0.044; *T* = 2.383); the quadratic term of temperature was also significant (*P* = 0.049), but the *T* value was 2.319—less than in the case of the linear term. This indicated that the linear term of temperature was more significant than the quadratic term of temperature.

(Insert Table 7)

On the basis of the results of the regression analysis, a second-order quadratic model was proposed, representing the empirical relationship between NDMA FP_{O3}/NDMA FP_{O3maximum} and the variables pH and temperature:

$$\begin{aligned} \text{NDMA FP}_{\text{O}_3}/\text{NDMA FP}_{\text{O}_3\text{maximum}} (Y) = & -8.807 + 1.956 \text{ pH} \\ & + 0.091 \text{ temperature} - 0.109 \text{ pH}^2 \\ & - 0.002 \text{ temperature}^2 \end{aligned} \quad (2)$$

3.3. Diagnostic checking of the fitted model

Analysis of variance was performed to check the adequacy and significance of the quadratic model in terms of larger F and low P value. The results of the ANOVA are predicted in Table 8. The P values for all of the variables were 0.05, indicating that the regression equation was significantly correlated with the response variable in the interpretation of the regression analysis. The significantly high value of F ($= 10.35$) indicated that the second-order polynomial model response ratio in equation 2 was adequate to represent the actual relationship between the response (*i.e.* the NDMA $\text{FP}_{\text{O}_3}/\text{NDMA}$ $\text{FP}_{\text{O}_3\text{maximum}}$) and the model process variables, namely pH and temperature.

(Insert Table 8)

The residuals were analyzed with the help of residual plots to determine whether the model met the assumption of the analysis. The normal probability plot of the residual values of the NDMA $\text{FP}_{\text{O}_3}/\text{NDMA}$ $\text{FP}_{\text{O}_3\text{maximum}}$ is shown in Fig. 5. The residual points on the plot fell close to the straight line representing a normal distribution of the residual [28].

(Insert Figure 5)

3.4. Analysis of 3D and 2D plots of the reaction curve of the model

The three-dimensional response surface plot, which is a 3D graphic representation of the regression equations, shows the individual and cumulative effects of the variables and the mutual interactions between the variables (Fig. 6). This figure indicates the quadratic nature of the surface with the maxima and minima of the response and the significance of the coefficient of the canonical equation. Whereas an isoresponse contour plot is a projection of the response surface on a 2D plane, a contour plot gives a better understanding than a 3D surface plot of the influence of variables and their interaction on the response [29].

(Insert Figure 6)

Fig. 7 is a contour plot representing the combined effect of pH and temperature on the NDMA $FP_{O_3}/NDMA FP_{O_3\text{maximum}}$, shown as absorbance. The maximum NDMA $FP_{O_3}/NDMA FP_{O_3\text{maximum}}$ occurred at pH 8.95 and temperature 20 °C.

(Insert Figure 7)

3.5. Optimum conditions for NDMA $FP_{O_3}/NDMA FP_{O_3\text{maximum}}$

A response optimization plot was drawn to identify the factor settings that optimize a single response or a set of responses. This is useful in determining the operating conditions that will result in a desirable response. The optimum conditions, which are defined as the best combination of factor settings for achieving the optimum response, were a temperature of 20.0 °C and a pH of 8.99 for a predicted response of absorbance of 0.90 and a desirability score of 0.99 (Fig. 8). There are many other advantages of using optimization plots, i.e., to achieve a predicted response with a higher desirability score; to lower cost factor settings with near-optimum properties; to study the sensitivity of response variables; to change factor settings; and to obtain the required response factor setting of interest.

(Insert Figure 8)

4. Conclusion

First, an attempt to reduce the concentrations of eight *N*-nitrosamines revealed that none was removed by ozonation in Milli-Q water for 2 h. Second, we optimized the basic experimental ozonation conditions (pH and temperature) for *N*-nitrosamine production by using a CCD method in wastewater matrices. The conditions were optimized, but of the eight *N*-nitrosamines only NDMA was produced. Therefore, either the precursors of the other *N*-nitrosamines were not present in the samples, or conditions other than pH 9 and a temperature of 20 °C were not suitable for producing *N*-nitrosamines other than NDMA. The maximum NDMA FP_{O_3} occurred at pH 8.96 and a temperature of 20.0 °C.

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Fig. 1. Semi-batch reactor used for the ozonation experiment.

Fig. 2. Concentrations of *N*-nitrosamines in Milli-Q (ozone feed rate, 3.1 mg/L/min; temperature, 20 °C; pH, 9; retention time, 120 min; 400 ng/L standard solutions).

Fig. 3. Pretreatment process flow chart for the eight *N*-nitrosamines and their three internal standards.

Fig. 4. Compound structures and GC-MS/MS chromatograms for the eight *N*-nitrosamines (250 µg/L standard solution; Rt: retention time).

Fig. 5. Normal probability plot of the difference between the observed and predicted values.

Fig. 6. 3D plot of the cumulative effects of pH and temperature on the NDMA $FP_{O_3}/NDMA FP_{O_3 \text{ maximum}}$.

Fig. 7. Contour plot of the cumulative effect of pH and temperature on the NDMA $FP_{O_3}/NDMA FP_{O_3 \text{ maximum}}$.

Fig. 8. Plot for optimization of variables (pH and temperature) for maximum production of NDMA FP_{O3}/NDMA FP_{O3maximum}.

Table 1

Ozonation experimental conditions by temperature, pH, reaction time, and ozone feed rate.

Researchers	Temp. (°C)	Optimum pH	Reaction time (min)	Ozone feed rate (mg/min/L)
Andrzejewski <i>et al.</i> [10], Poland, UK	20	10.5 from 6.5, 7.5, 8.5, 9.5, 10.5	60	0.4
Schmidt <i>et al.</i> [11], Germany	21	7	30	6 ^a
Oya <i>et al.</i> [12–14], Japan	20	7	20	10
Kosaka <i>et al.</i> [16], Japan	20	7	15	10
Zhao <i>et al.</i> [17], Canada	20	7	10	10
Chen <i>et al.</i> [18], Chnia	25	10 from 2.5, 3, 5.6, 7, 8.7, 10, 11.6	.	0.67 ^a

^a mg/L (Only ozone concentration)

Table 2Characteristics of target *N*-nitrosamines, and optimized analytical parameters.

No.	<i>N</i> -nitrosamine	CAS number	Molecular weight	Corresponding internal standard	MS parameters		
					PI ^a (<i>m/z</i>)	DI ^b (<i>m/z</i>)	CE ^c (eV)
1	<i>N</i> -nitrosodimethylamine (NDMA)	62-75-9	74.08	NDMA- <i>d</i> ₆	75	43 58	15 10
2	NDMA- <i>d</i> ₆ (internal standard)	17829-05-9	80.12	·	80	46 64	20 10
3	<i>N</i> -nitrosomethylethylamine (NMEA)	10595-95-6	88.11	NPYR- <i>d</i> ₈	89	43 61	10 5
4	<i>N</i> -nitrodiethylamine (NDEA)	55-18-5	102.14	NPYR- <i>d</i> ₈	103	56 75	20 5
5	<i>N</i> -nitrosodi- <i>n</i> -propylamine (NDPA)	621-64-7	130.22	NDPA- <i>d</i> ₁₄	131	43 89	10 5
6	NDPA- <i>d</i> ₁₄ (internal standard)	93951-96-3	145.20	·	145	97	5
7	<i>N</i> -nitrosopyrrolidine (NPYR)	930-55-2	100.11	NPYR- <i>d</i> ₈	101	55 70	10 5
8	NPYR- <i>d</i> ₈ (internal standard)	1219802-09-1	109.12	·	109	62	10
9	<i>N</i> -nitrosopiperidine (NPIP)	100-75-4	114.15	NPYR- <i>d</i> ₈	115	41 69	15 10
10	<i>N</i> -nitrosomorpholine (NMOR)	59-89-2	116.10	NPYR- <i>d</i> ₈	117	57 87	10 5
11	<i>N</i> -nitrosodi- <i>n</i> -butylamine (NDBA)	924-16-3	158.24	NDPA- <i>d</i> ₁₄	159	57 103	10 5

^a Parent ion. ^b Daughter ion. ^c Collision energy.

CAS, Chemical Abstracts Service.

Table 3

Characteristics of wastewater treatment plant N.

Service area (km ²)	Average flow (m ³ /day) ^a	Service population	SRT ^b (days)	Secondary treatment	Disinfection
18,69	288,000	292,500	3.8	CAS	NaClO

^a Average dry weather value during the study period^b Sludge retention time^c Conventional activated sludge**Table 4**

Characteristics of influent properties at wastewater treatment plant N.

pH	BOD (mg/L)	COD ^a (mg/L)	SS (mg/L)	T-N (mg/L)	NH ₄ -N (mg/L)	NO ₂ -N (mg/L)	NO ₃ -N (mg/L)	T-P (mg/L)
7.4	120	75	120	34.8	28	0.01	0.05	2.5

^a COD_{Mn}**Table 5**

Detailed analytical parameters for GC-MS/MS.

GC chromatograph (Varian 450-GC)	
Column	VF-17 ms, 30 m × 0.25 mm ID
Flow rate	1 mL/min
Oven program	40 °C, hold 1 min, To 80 °C at 5 °C/min, To 280 °C at 20 °C/min, hold 3 min
Injector temperature	250 °C
Injection mode	Splitless, hold 1 min
Injection volume	2 µL
Mass spectrometer (Varian 300-MS)	
Ionization mode	Positive chemical ionization
Detector range	600 to 2000 V
Source temperature	220 °C
Transfer line temperature	250 °C
Carrier gas	Helium at 1.5 mL/min
CI gas	Methane
Filament Current	50 µA
Electron energy	70 eV

Table 6

Predicted and experimental data showing NDMA $FP_{O_3}/NDMA\ FP_{O_3\text{maximum}}$ at different pH and temperature ($^{\circ}C$).

Temperature ($^{\circ}C$)	pH	Experimental value	Predicted value	Residual
10	7	0.17	0.2277	-0.0558
10	11	0.42	0.1884	0.2299
30	7	0.34	0.3010	0.0341
30	11	0.31	0.2618	0.0519
20	7	0.50	0.4831	0.0218
20	11	0.16	0.4439	-0.2818
10	9	0.47	0.6449	-0.1740
30	9	0.63	0.7183	-0.0859
20	9	0.95	0.9004	0.0520
20	9	0.90	0.9004	0.0043
20	9	0.93	0.9004	0.0282
20	9	1.00	0.9004	0.0996
20	9	0.98	0.9004	0.0758

Table 7

Estimated regression coefficients of NDMA $FP_{O_3}/NDMA\ FP_{O_3\text{maximum}}$ versus pH and temperature, in uncoded units.

Term	Coeff.	SE of coeff.	<i>T</i>	<i>P</i>
Constant	-8.807	1.795	-4.907	0.001
pH	1.956	0.426	4.596	0.002
Temperature	0.091	0.038	2.383	0.044
pH $\times$ pH	-0.109	0.023	-4.632	0.002
Temperature $\times$ temperature	-0.002	0.001	-2.319	0.049

$R^2 = 83.8\%$, R^2 (adjusted) = 75.7%

Table 8

Analysis of variance of the NDMA $FP_{O_3}/NDMA\ FP_{O_3\text{maximum}}$ versus pH and temperature, in uncoded units.

Source	DF ^a	Seq. SS ^b	Adj. SS ^c	Adj. MS ^d	<i>F</i> ^e	<i>P</i> ^f
Regression	4	1.017	1.017	0.254	10.35	0.003
Linear	2	0.010	1.000	0.500	20.36	0.001
Square	2	1.007	1.007	0.503	20.48	0.001
Residual error	8	0.197	0.197	0.025		
Total	12	1.213				

^a Degrees of freedom

^b Sequential sum of squares

^c Adjusted sum of squares

^d Adjusted mean square

^e Mean square due to regression (MSR)/mean square due to error (MSE)

^f Defined as the smallest level of significance leading to rejection of the null hypothesis