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Multivariate Statistical Analysis of Antioxidants in Dates (*Phoenix dactylifera*)

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Abstract: Four types of soft dates (SD), three types of semi-dried dates (SDD) and one type of dried dates (DD) were used in this study. The antioxidant activities were assessed using TEAC method (ABTS assay) and the ferric reducing/antioxidant power method (FRAP assay), while total phenolic content (TPC) and total flavonoid content (TFC) were measured using Folin-Ciocalteau and aluminum chloride colorimetric methods. Multivariate analysis of variance (MANOVA), discriminant analysis (DA) and principal component analysis (PCA) were used to analyze the data. MANOVA showed a strong significant difference between the eight types of dates. DA identified the relative contribution of each parameter in distinguishing the dates. DA also identified two functions responsible for discriminating the dates and showed the difference between different types of dates. The first function distinguished DD from other types of dates, whilst the second function discriminated SD and SDD, affording 100% correct assignation. PCA identified only one component responsible for explaining 98.85% of the total variance in antioxidant data. It is suggested that the TEAC method and the quantitative determination of TPC and TFC was suitable for differentiation of dates and quality control.

Keywords: MANOVA, principal component analysis, discriminant analysis, dates, total phenolic content, total flavonoid content

INTRODUCTION

Dates have an important antioxidant activity due to the presence of phenolic and flavonoid compounds (Mansouri *et al.*, 2005; Biglari *et al.*, 2008). Various factors such as variety, growing conditions, maturity, season, geographic origin, fertilizer, soil type, storage conditions, and amount of sunlight received, have been reported to be responsible for the differences in antioxidants of dates (Al-Farsi *et al.*, 2007). Assessment of antioxidant activities in dates may be performed using TEAC method (ABTS assay) and the ferric reducing/antioxidant power method (FRAP assay), while the content of phenolic and flavonoids compounds may be quantified using Folin-Ciocalteau and aluminum chloride colorimetric methods, respectively (Biglari *et al.*, 2008). Differentiation of dates based on antioxidant activity and antioxidative compounds may be beneficial for commercialization purposes. Varieties of dates with higher antioxidant activity and antioxidative compounds may be processed into higher value-added products such as functional food ingredients or nutraceuticals.

The application of multivariate statistical methods has increased tremendously in recent years for analyzing food data (Rosa *et al.*, 2006; Nilva *et al.*, 2006; Dirk, 2007). Multivariate methods are useful where several dependant variables are measured on each sampling unit. Multivariate statistical analysis can provide more information than univariate statistical techniques or descriptive statistics in terms of explaining the sources of variation in data. The differences in antioxidative compounds and antioxidant activity between all types of dates can be analyzed and interpreted using multivariate statistical techniques. Such analysis has not been performed on antioxidant activity and antioxidative compounds of dates from Iran. In our study, three statistical techniques were used, namely multivariate analysis of variance (MANOVA) which tested the significant differences, while discriminant analysis (DA) was applied to identify the relative contribution of all variables to the separation of the groups. Principal component analysis (PCA) is a data reduction technique used in distinguishing the number of significant variates. PCA was applied to explain the observed variances in the data and to understand the interrelationship between different parameters (Richard and Dean, 2002; Alvin, 2002).

The objective of this study was to apply multivariate statistical techniques to test the differences among the selected varieties of dates from Iran, in terms of antioxidant activities (ABTS

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and FRAP assay) and antioxidative compounds (total phenolic and flavonoids content). The statistical techniques were also used to detect the effects of each parameter on the degree of differentiation among the same variety of dates and to identify the source of variation in the dates.

MATERIALS AND METHODS

Plant Material

Fresh ripe date samples used in the experiments consisted of four types of soft dates (SD) locally known as Honey, Bam, Jiroft and Kabkab dates, three types of semi-dried dates (SDD) locally known Sahroon, Piarom and Zahedi dates, and one type of dried date (DD) locally known as Kharak date. Dates ripen in four stages, which are known throughout the world by their Arabic denominations; kimri (unripe), khalal (full-size, crunchy), rutab (ripe, soft) and tamr (ripe, reduced moisture). The date goes from one extreme at moisture content of 85% at early Kimiri stage to 50-60% for Khalal, 35-40% for Rutab, and 20% for Tamr. Due to differences in variety and growth conditions, dates vary in shape, size, weight and moisture content. The practical sub-division of dates into soft dates (SD), semi-dried dates (SDD) and dried dates (DD) was based on their external qualities of texture, pliability and the ratio between glucose, fructose and sucrose content at the tamr stage (Biglari *et al.*, 2008). All samples were procured at the beginning of the 2006 harvest season. Identical samples based on size, color, ripening stage, without damage and calamity from a dates distribution center in the capital city of Tehran and were transported in paper bags in a refrigerator to Malaysia for the studies. Each date weighed about 7 to 10 g per fruit and for each extraction, approximately 100 g (~ 10 dates) of each type of date was used. Three replications were used with 10 dates per replicate for each type of date. The experimental units were carefully selected from the main distribution center on different days.

Chemicals and Reagents

The compounds 2,2 2-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,4,6-tripyridyl-1,3,5-triazine (TPTZ) were purchased from Sigma Chemical Company, USA. $FeCl_3 \sum 3H_2O$, potassium persulphate, sodium acetate, and sodium carbonate were obtained from Sigma/Aldrich. Folin–Ciocalteu reagent, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Merck. All Chemicals and reagents used were of analytical grade.

Sample Preparations and Analysis

The flesh of the date (100 g) was pitted, crushed and cut to small pieces and dry-blended for 3 minutes (Panasonic, Malaysia). The extraction solvent was 300 ml of methanol–water (4:1, v/v), and extraction was performed at 20°C for 5 hrs using an orbital shaker. Each date weighed approximately 7 to 10 g per fruit and for each extraction 100 g of each type of date was used (Biglari *et al.,* 2008). The extracts were then filtered and centrifuged (Hettich Zentrifugen) at 4000 *g*, for 10 min and the supernatant was concentrated under reduced pressure at 40°C for 3 hr using a rotary evaporator (IKA- WERKE-RV06ML) to obtain date palm fruit (DPF) methanolic crude extract. The crude extract was kept in dark glass bottles for three days inside a freezer (SANYO, Japan) until use.

Analysis for TPC and TFC were performed following the methods described by Biglari *et al.* (2008). The TPC analysis was based on the methods described by Singleton and Rossi (1965) using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) and Folin ciocalteau reagents. The optical density of the blue-coloured samples was measured at 765 nm. The total phenolic contents were expressed as mg gallic acid equivalent (GAE)/100g dry basis.

The determination of flavonoids was performed according to the colorimetric assay of Kim *et al.* (2003) as described previously (Biglari *et al.*, 2008). A calibration curve was prepared with catechin and the results were expressed as mg catechin equivalents (CEQ)/100g dry sample. Each sample was independently extracted in triplicates, and analyses were performed on the same day.

Antioxidant activities of the methanolic extract of dates were assessed using ABTS and FRAP assays as described by Biglari *et al.* (2008). For the ABTS assay, a standard curve was obtained by using Trolox standard solution at various concentrations (ranging from 0 to 15 μM) in 80% ethanol. The absorbance of the reaction samples was compared to that of the Trolox standard and the results were expressed as Trolox equivalent (Re *et al.*, 1999). The FRAP assay was based on the modified method of the assay of ferric reducing/antioxidant power (FRAP) of Benzie and Strain (1999). Aqueous solutions of known Fe (II) concentrations in the range of 100–2000 μM ($FeSO_4 \sum 7H_2O$) were used for calibration (Biglari *et al.*, 2008).

STATISTICAL ANALYSIS

Multivariate Analysis of Variance (MANOVA)

Multivariate analysis of variance was used where several dependent variables (*p*) were measured on each sampling unit instead of one variable. The objective of using MANOVA was to compare the mean vectors of *k* groups for significant differences. Equality of the mean vectors implies that the *k* means are equal for each variable, where two means with only one different variable will be considered having different mean vectors of the *k* groups (Alvin, 2002; Bryan, 1991; Richard and Dean, 2002).

Discriminant Function

Discriminant analysis is a multivariate technique used for two purposes; 1) to describe group separation in which linear functions of the several variables (discriminant functions (DFs)) are used to describe or elucidate the differences between two or more groups, leading to the identification of the relative contribution by all variables prior to groups separation, 2) to predict or allocate observations in which linear or quadratic functions of the variable (classification functions (CFs)) are used to assign an observation to one of the groups (Alvin, 2002; Bryan, 1991; Richard and Dean, 2002).

Principal Component Analysis (PCA)

Principal component analysis (PCA) is designed to transform the original variables into new uncorrelated variables called components, which are linear combinations of the original variables. The PCA is a data reduction technique used in determining the number of variates to explain the observed variances in the data (Alvin, 2002; Bryan, 1991; Richard and Dean, 2002).

RESULTS AND DISCUSSION

Descriptive Statistics

Descriptive statistics including the minimum, maximum, mean and standard deviation (Std) are shown in Table 1. Kharak dates exhibited the highest values of Total Flavonoid content (TFC), Total Phenolic contents (TPC), TEAC and FRAP, whilst Jiroft dates exhibited the lowest values of these parameters. The maximum values of TFC, TPC, TEAC and FRAP were 79.58 (mgCEQ/100g dw), 123.87 (mgGAE/100g dw), 329.45 (μmol/100 g dw), 594.71 (μmol Trolox equivalents /100g dw), respectively and the minimum values were 0.85 (mgCEQ/100g dw), 1.57 (mgCEQ/100g dw), 6.96 (μmol/100 g dw), 11.82 (μmol Trolox equivalents/100g dw), respectively. The spread around the mean was small for all parameters except TEAC which exhibited a large fluctuation with most types of dates. In general, all types of dates exhibited reasonable difference between the maximum and minimum values except Kharak dates that did not show consistent differences. This indicated that Kharak dates exhibited extreme values compared to other types of dates. Correlation matrix for TFC and TPC contents in dates and FRAP and TEAC was also examined (Table 2). A strong positive relationship was shown between all parameters, indicating that the parameters have a strong association among them and shared a common source.

Multivariate Analysis

The data was analyzed using univariate statistical method such as simple linear regression (Biglari *et al.*, 2008). The results of multivariate analysis of variance (MANOVA) for TFC, TPC, FRAP and TEAC in dates are given in Table 3. Based on these results, the contents of antioxidative compounds in different types of dates exhibited a strong significant difference in terms of selected parameters. The data was further evaluated by using discriminant analysis (DA). The DA was applied on the raw data consisting of four variables. Four discriminant functions (DFs) were found to discriminate the eight types of dates. Wilk's Lambda test showed that only the first two DFs are statistically significant as shown in Table 4. The relative contribution for each parameter is given in Eq. 1 and Eq. 2.

$$Z_1 = -2.52\ TFC + 2.22\ TPC + 0.68\ FRAP + 2.99\ TEAC \quad (1)$$

$$Z_2 = -6.25\ TFC + 4.59\ TPC + 1.30\ FRAP + 5.29\ TEAC \quad (2)$$

It is clear that TEAC exhibited the highest contribution in discriminating the types of dates (Eq.1), followed by TFC and TPC, and account most of the expected variations in the selected parameters, whilst FRAP showed less contribution in explaining the variations between the eight types of dates. The second DF exhibited different contributions from the first DF, since TFC exhibited the highest contribution, followed by TEAC and TPC, while FRAP contributed the lowest. The result from this analysis was reasonable since the reaction time of the improved ABTS assay was only 6 min, while the FRAP assay which measures the reducing capability by increased

Table 1: Descriptive statistics including minimum, maximum, mean and standard deviation (sd) for selected parameters of the dates

Type of Date	Parameter	Minimum	Maximum	Mean	sd
Honey	TFC	1.64	1.82	1.73	0.088
	TPC	2.66	2.77	2.72	0.056
	FRAP	10.06	11.96	11.28	1.06
	TEAC	21.16	23.04	22.23	0.97
Kabkab	TFC	1.01	1.25	1.09	0.14
	TPC	2.00	2.34	2.19	0.18
	FRAP	7.47	8.52	7.83	0.59
	TEAC	22.26	34.85	27.04	6.82
Bam	TFC	1.64	1.96	1.79	0.16
	TPC	2.10	2.36	2.23	0.13
	FRAP	9.66	11.04	10.24	0.71
	TEAC	18.00	19.43	18.95	0.82
Jiroft	TFC	0.85	1.04	0.93	0.10
	TPC	1.57	1.62	1.60	0.03
	FRAP	6.96	7.87	7.38	0.46
	TEAC	11.82	25.03	16.78	7.19
Piaron	TFC	3.03	3.90	3.41	0.44
	TPC	4.27	4.56	4.41	0.14
	FRAP	19.56	22.40	21.21	1.47
	TEAC	34.60	43.41	38.55	4.47
Sharoon	TFC	0.96	1.63	1.192	0.38
	TPC	4.73	4.85	4.81	0.06
	FRAP	18.44	21.08	19.46	1.42
	TEAC	31.88	35.82	34.40	2.20
Zahedi	TFC	3.36	4.71	3.88	0.72
	TPC	3.18	3.32	3.23	0.10
	FRAP	13.82	14.63	14.14	0.43
	TEAC	25.80	44.23	35.530	9.26
Kharak	TFC	55.98	79.58	69.29	12.09
	TPC	117.18	123.87	119.75	3.61
	FRAP	326.30	329.45	328.14	1.64
	TEAC	397.57	594.71	465.48	111.97

Table 2: Linear correlation coefficient matrix for selected parameters in the dates

Parameter	TFC	TPC	FRAP	TEAC
TFC	1			
TPC	0.99**	1		
FRAP	0.99**	0.99**	1	
TEAC	0.99**	0.97**	0.97**	1

**Correlation is significant at the P< 0.01.

Table 3: Multivariate analysis of variance (MANOVA) for the eight types of dates

Test	Value	F	P-value
Pillai's Trace	2.04	2.39	<0.002
Wilks' Lambda	0.00	58.06	<0.001
Hotelling's Trace	25619.94	10522.48	<0.001
Roy's Largest Root	25608.61	58533.95	<0.001

Table 4: Wilks' Lambda for testing discriminant function validity

Test of Function(s)	Wilks' Lambda	P-value
1 through 4	0.00	<0.001
2 through 4	0.07	<0.001
3 through 4	0.88	0.99
4	0.99	0.99

Table 5: Classification results for discriminant analysis of all types of the dates

Type of date	% correct[a]	Predicted Group Membership							
		Honey	Sharoon	Bam	Jiroft	Piarom	Kabkab	Zahedi	Kharak
Honey	100	3	0	0	0	0	0	0	0
Sharoon	100	0	3	0	0	0	0	0	0
Bam	100	0	0	3	0	0	0	0	0
Jiroft	100	0	0	0	3	0	0	0	0
Piarom	100	0	0	0	0	3	0	0	0
Kabkab	100	0	0	0	0	0	3	0	0
Zahedi	100	0	0	0	0	0	0	3	0
Kharak	100	0	0	0	0	0	0	0	3

[a]100.0% of original grouped cases correctly classified.

sample absorbance based on the ferrous ions released may have utilized a longer period. Therefore the assay may not be completed even for several hours upon initiation of the reaction, such that a single end-point of the reaction cannot be determined (Prior *et al.*, 2005). The FRAP assay may also suffer from drawbacks such as interference, reaction kinetics, and quantitation method (Ou *et al.*, 2002). Based on these statistical analyses, we suggest that the TEAC method, quantitative determination of TPC and TFC could be considered reliable for the determination of dates differentiation, and as a measure for quality control for the assessment of antioxidants.

The relationship between the samples of different dates and the scores of the two discriminant functions are presented in Figure 1. It is clear that the first DF was responsible for discriminating Kharak date from other types of dates, while the second DF was responsible for discriminating other types of dates without discriminating Kharak from other types of dates. The classification results (Table 5) showed that 100% of the cases were correctly classified to their respective type. The results of classification showed that the significant differences that existed between these types of dates were expressed in terms of the two DFs.

Principal components analysis (PCA) was carried out on the data (4 variables) to compare the compositional patterns between the analyzed selected parameter samples and to identify the sources of variation. PCA yielded one component with Eigen-value >1, explaining 98.85% of the total variance in the data set. An Eigen-value gives a measure of the significance of the component; the component with the highest Eigen-value is the most significant and responsible in explaining large variation in the data. The Eigen-values for different components, percentage variance accounted, and cumulative percentage variances are given in Table 1. The PCA was actually performed on the correlation matrix between different parameters.

The parameter loadings for the components yielded from the PCA of the data are given in Eq. 3.

$$PCA = 0.99\ TFC + 0.99\ TPC + 0.99\ FRAP + 0.99\ \text{TEAC} \quad (3)$$

The above Eq. 3 accounted for 98.85% of the total variance and was positively correlated with TFC, TPC, FRAP and TEAC. This component may be termed as the average of all selected parameters, since all parameters contributed highly in explaining the total variation in the data. Extracting only one component indicated that the parameters were highly inter-correlated.

The relationship between component scores and the samples from different types of dates were studied to understand the behavior of selected parameters. Component scores for different types of dates are given in Figure 2. It can be seen that SD and SDD contributed negatively while DD contributed positively to the scores. This difference in contribution supported the results obtained by DA, indicating that DD had different characteristics from SD and SDD.

All multivariate statistical techniques indicated that the eight types of dates had different antioxidant activity and contents of antioxidative compounds. In general, more information was obtained by using the multivariate compared to univariate statistical techniques or descriptive statistics in terms of explaining the sources of variance in the data (PCA), and in terms of

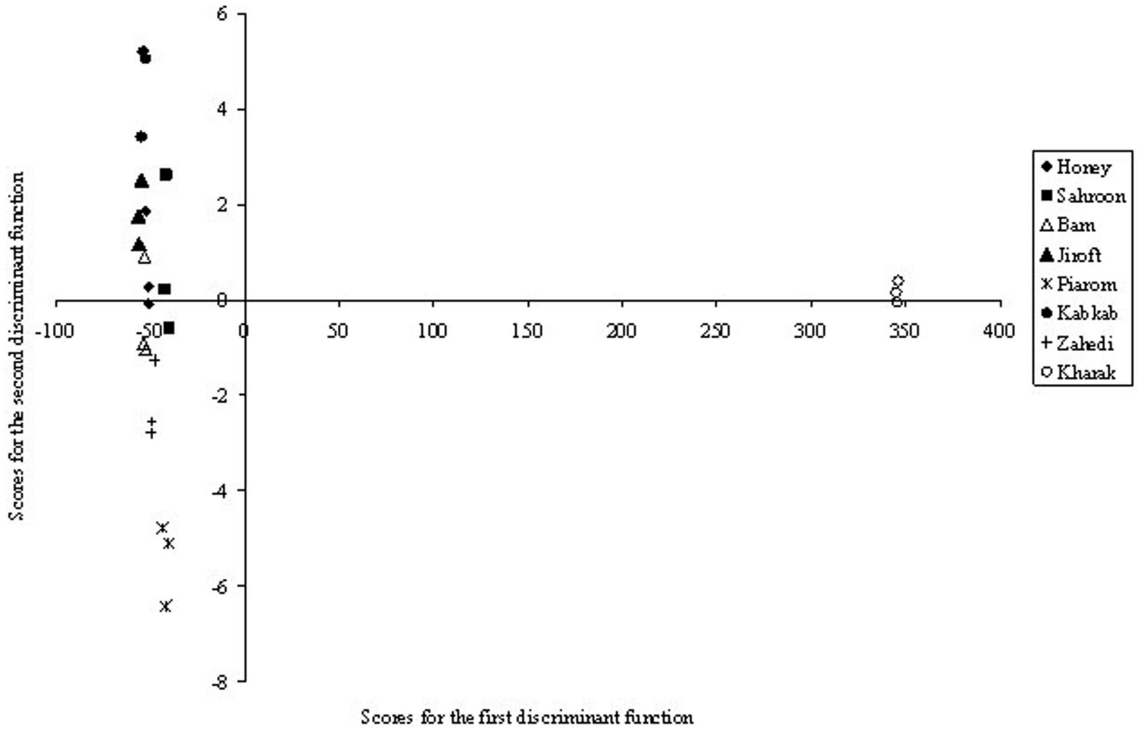

Figure 1: First and second discriminant functions for all types of the dates

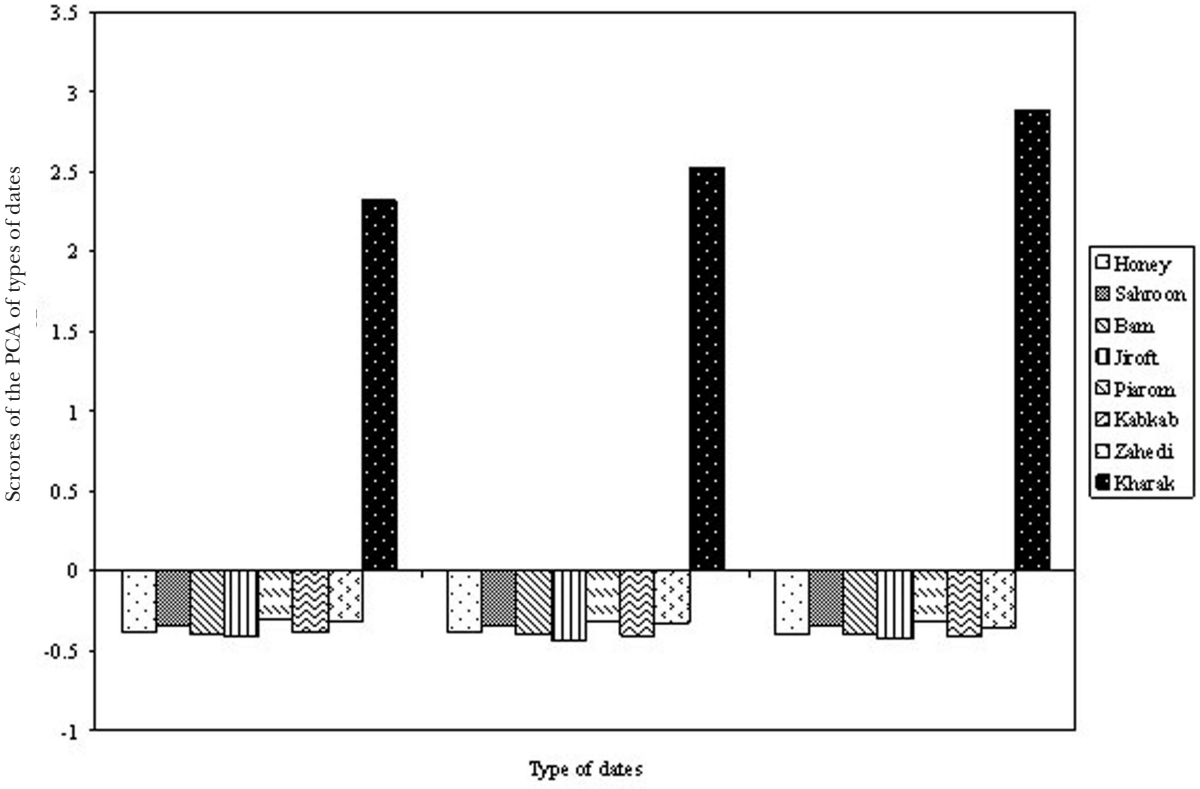

Figure 2: Scores of the PCA for all types of the dates

Table 6: Extracted values of PCA for all types of the dates

	Total variance explained		
	Extraction Sums of Squared Loadings		
Component	Total	% of Variance	Cumulative %
1	3.954	98.85	98.85

understanding the differences between all types of dates especially the difference between DD and other types of dates as explained by DA and also exhibited by PCA. Some inconsistencies were noted in the values of antioxidant activity and levels of antioxidative compounds from the same types of dates. These might be attributed to sampling methods employed such as collecting dates on different days, and due to differences in the analytical methods that have different mechanisms of action or different reaction conditions.

CONCLUSION

From the above discussion and multivariate results, it can be concluded that the total variance was explained by the average of all parameters included in the analysis as described by PCA. In addition, DD exhibited a strong difference compared to the other types of dates studied, indicating a different content of antioxidative compounds as supported by both PCA and DA. DA identified two functions responsible for distinguishing between different types of dates. The first function was responsible for distinguishing DD from other types of dates, whilst the second function was responsible for discriminating SD and SDD, affording 100% correct assignation.

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