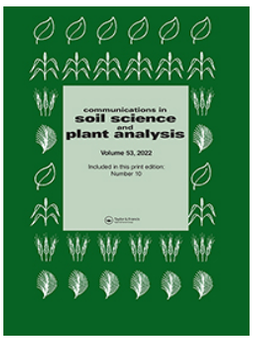

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A New Extractant for Concurrent Estimation of Nicotine, Reducing Sugars and Chlorides in Tobacco Cured Leaf

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ABSTRACT

The objective of the study is to develop simple, inexpensive, and less hazardous extractant for simultaneous extraction of nicotine, reducing sugars, and chlorides in tobacco cured leaf. The currently used reference extractant for nicotine, reducing sugars, and chlorides comprises 20% aqueous methanol +5% acetic acid+2 cc charcoal in 15:4:1 ratio. As the methanol, a major component of this reference extractant, is hazardous and expensive, we proposed and evaluated a simple extractant involving 10%acetic acid for assaying nicotine, reducing sugars, and chlorides in comparison to reference extractant. The sample data (n = 30) sets of both the extractants [proposed (x) and reference (y)] were subjected to Passing &Bablok's regression analyses. The regression models so obtained for reducing sugars (y = 0.594 + 1.021x), nicotine (y = 0.044 + 0.8877x), and chlorides (y = 0.057 + 1.131x) were validated by CUSUM test for linearity. The test indicated that there was no significant deviation from linearity for nicotine, reducing sugars, and chlorides. Spearman's rank correlation coefficient (ρ) with P < .0001at 95% confidence interval also showed high correlation between the proposed and the reference extractants for Nicotine (0.993), reducing sugars (0.982), and chlorides (0.972). Analyses of the data sets by Youden plot method showed congregation of data points near to diagonal reference line and a few wild points. This type of data points distribution indicates the high degree of comparability between the proposed and the reference extractants for nicotine, reducing sugars, and chlorides in tobacco cured leaf. The estimated cost of extraction was less with the proposed extractant. It is suggested that the proposed extractant (10%acetic acid + 4 cc activated charcoal suspension) being simple and less expensive be used as an alternative to the hazardous methanol-based extractant.

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KEYWORDS
Non-methanol extractant; 10% acetic acid; tobacco leaf; nicotine; reducing sugars; chlorides

Introduction

Tobacco (*Nicotiana tabacum* L.) is the most widely grown commercial non-food plant. Tobacco leaf quality as gauged by nicotine, reducing sugar, and chloride content plays a vital role in determining its market price. The quality constituents of cured leaf viz., nicotine, reducing sugar, and chloride are generally extracted with organic solvents such as methanol, ether, ethanol, acetic acid, and other organics (Harvey, Stahr, and Smith 1969; Ngozi Donald 2020). Alkaloids can be removed from aqueous basic solution with organic solvents such as chloroform, ether, methylene chloride, and other organics that are immiscible with water (Wilkinson and Weeks (1994). The extractant involving 20% aqueous methanol+5% acetic acid+2 cc charcoal in 15:4:1 ratio (Harvey, Stahr, and Smith 1969) is widely used for determining nicotine, reducing sugar, and chloride contents in tobacco cured leaf and is considered as a reference extractant.

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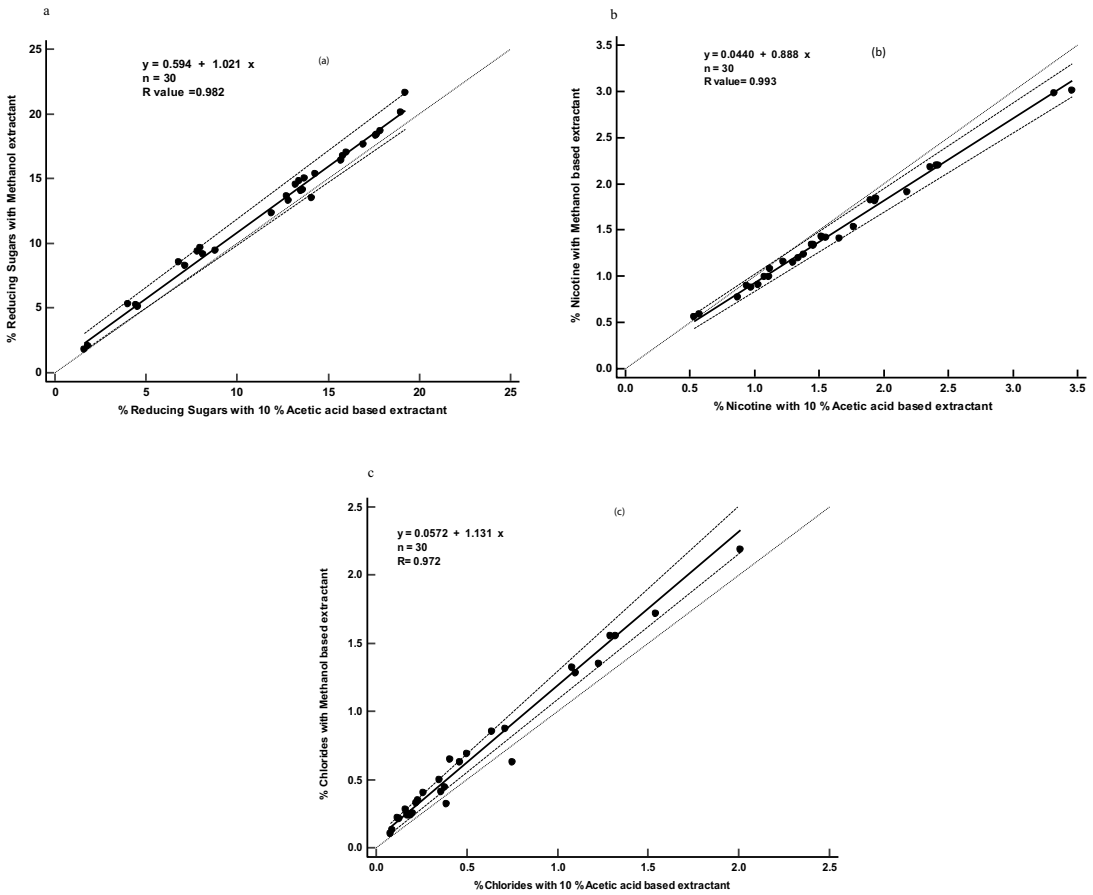

Figure 1. Regression of reducing sugars (a), nicotine (b), and chlorides (c) estimated in tobacco leaf samples by proposed and reference extractant (n = 30).

A major component of the reference extractant is methanol. Methanol, being a hazardous solvent (Finkelstein and Vardi 2002; Hageman, van der Hoek, and Faber 2003), has usage restrictions with a permissible exposure limit (PEL) of 200 ppm in the air for an eight-hour shift (National Institute for Occupational Safety and Health (NISOH 1978). It is also relatively more expensive than the other polar solvents. It is, therefore, important to identify nonhazardous extractant in place of currently used methanol based extractant (reference) for routine estimation of nicotine, reducing sugar, and chloride contents in tobacco-cured leaf. The present investigation, therefore, aims at evaluating a simple nonhazardous solvent as an extractant for nicotine, reducing sugar, and chloride in tobacco-cured leaf. A similar polar solvent, acetic acid with a polarity index of 6.2 was selected as an extractant and evaluated against the reference extractant.

Materials and methods

Sample and sample preparation: The appropriate sample size is important for comparison of two or more extractants or methods. A comparison of extractants/methods based on small sample sizes often results in a biased inference that the laboratory methods are in agreement. The width of the 95% confidence intervals for intercept and slope will be large when sample size is small, and therefore, large size of sample needs to be used. Bablok and Passing (1985) advocated to have at least 30 samples for

methods comparison. In the present study, a sample size of 30 was chosen for comparing two extractants used in assaying nicotine, reducing sugars, and chloride in tobacco-cured leaf. As the chemical composition of tobacco is known to vary from one geographical region to the another (Wilkinson and Weeks 1994) the tobacco cured leaf samples for this study (n = 30) were collected from different agro-climatic zones varying in soil types and management levels. Selecting samples in such a way that represents a broad range of analyte of interest is necessary for comparing extractants (Damodar Reddy and Krishnamurthy 2013).

The leaf lamina was separated, dried in oven, and powdered. The powdered leaf samples were sieved (< 0.5 mm sieve), labeled with codes and kept stored in closed butter paper bags until the analyses. Nicotine, reducing sugars, and chlorides in prepared tobacco-cured leaf samples were extracted by the reference and proposed extractants.

Extraction by reference extractant: A set of samples was used for extracting nicotine, reducing sugars, and chlorides in tobacco leaf samples by using standard extractant (reference) containing methanol (CH_3OH) + acetic acid (CH_3COOH) + water in the volume ratio of 4:1:15. To this a 2 cc activated charcoal suspension prepared by dissolving 135 g Darco-G in 450 ml of Glycerol and 450 ml of distilled water (Harvey, Stahr, and Smith 1969).

Extraction by proposed extractant: Another set of samples used for extracting nicotine, reducing sugars, and chlorides in tobacco leaf samples by using proposed extractant comprising 10% acetic acid + 4 cc activated charcoal suspension (prepared as above).

Extraction procedure

Two hundred fifty (250 mg) milligrams of tobacco leaf lamina powder sample was taken in 150 ml conical flask and 50 ml of either reference or proposed extractant was added to get a sample to extractant ratio of 1:200 ((w/v). The contents in the flask were shaken using rotary shaker for 15 minutes at 170 rpm for homogenous mixing. The extract was filtered into cuvettes using *Whatman no. 5* filter paper.

Estimation of nicotine, reducing sugars, and chlorides

The concentrations of nicotine, reducing sugars, and chlorides in the extracts obtained by the reference and proposed extractants were concurrently determined colorimetrically using Auto-Analyzer AA3 (Bran+Luebbe, Germany) as per the procedure outlined by Harvey *et al.*, 1958. In brief, nicotine in the extract was determined by developing color using cyanogen bromide and buffered aniline solution and measuring the color intensity at 460 nm. The concentration of reducing sugars in the extracts was quantified by using alkaline potassium ferricyanide (K_3 [Fe $(CN)_6$] and sodium chloride reagents for developing characteristic color and measuring the intensity of color at 420 nm. The chloride concentration in the extract was quantified by using color reagent comprising mercuric thiocyanate (Hg $(SCN)_2$) and ferric nitrate (Fe $(NO)_3$ $9H_2O$) and measuring color intensity at 480 nm.

Flow process of nicotine channel was fixed at 0.23 ml/min for sample, 0.32 ml/min for cyanogen bromide and 1.00 ml/min for buffer. While rate of flow was 0.10 ml/min for sample, 1.40 ml/min for K3 Fe (CN) 6, 1.00 ml/min for sodium salt solution, and 0.32 ml/min for air flow in case of reducing sugars. The sample, color reagent, diluent, and wash flow rates for chlorides were 0.10 ml/min, 1.00 ml/min, 1.40 ml/min, and 2.00 ml/min fixed, respectively.

The contents of nicotine, reducing sugars, and chlorides present in the tobacco-cured leaf samples were calculated and expressed in percentage as below.

Nicotine (%) or Reducing Sugars (%) or Chlorides (%) = $(0.005 \times P)/W$

Where, P = PPM of Nicotine or Reducing Sugars or Chlorides

W = Weight of the tobacco leaf powder in grams

Data analysis: The laboratory data for nicotine, reducing sugar, and chloride contents in tobacco leaf samples as obtained by the proposed extractant and the reference extractant were analyzed using relevant statistical techniques and methods for comparison, i.e. Passing & Bablok regression,

spearman rank correlation, CUMSUM test, and Youden plot method. The XLSTAT statistical and data analysis solution, Addinsoft (2021) and MedCalc® Statistical Software version 19.6.3., (2021) were employed for data analyses.

Results and discussion

Nicotine, reducing sugars, and chloride contents in tobacco cured leaf

The details of descriptive statistics and statistical results on comparison of two extractants for estimation of nicotine, reducing sugars, and chlorides in tobacco cured leaf are presented in Table 1a & Table 1b. Nicotine content in 30 tobacco leaf samples varied from 0.53 to 3.46% with the proposed extractant and 0.56 to 3.01 with reference extractant. Reducing sugar content in leaf samples ranged from 1.61 to 19.24% with the proposed extractant and from 1.81 to 21.63% with the reference extractant. The leaf chloride content varied from 0.08 to 2.01% in case of proposed extractant, while it ranged from 0.10 to 2.19% with reference extractant.

The sample data of the two sets belonging to the reference extractant and the proposed extractant was subjected to Passing and Bablok (1983) regression to compare and evaluate the relationship between the determined concentrations using the proposed extraction and the reference (Table 1a & b). The regression equation with the calculated values for intercept and slope according to Passing and

Table 1a. Comparison of values (n = 30) of test variables obtained by the reference (A) and the proposed (B) extractants.

	Nicotine		Reducing sugars		Chloride	
Sample	A	B	A	B	A	B
1	1.91	2.18	1.81	1.61	2.19	2.01
2	0.77	0.87	2.05	1.81	0.50	0.35
3	3.01	3.46	5.12	4.55	0.32	0.39
4	1.43	1.52	5.28	4.03	1.35	1.23
5	0.90	0.94	8.26	7.12	0.44	0.38
6	1.16	1.22	8.52	6.78	1.55	1.29
7	1.81	1.93	9.15	8.13	0.24	0.18
8	0.99	1.11	9.40	7.83	1.32	1.08
9	1.42	1.55	9.64	7.99	0.65	0.41
10	0.99	1.08	12.31	11.89	0.21	0.13
11	1.34	1.44	13.26	12.80	0.28	0.16
12	1.43	1.52	13.52	14.11	0.10	0.08
13	2.20	2.42	13.64	12.70	0.25	0.17
14	1.23	1.38	14.08	13.49	0.22	0.12
15	0.59	0.57	14.79	13.42	0.85	0.64
16	1.33	1.46	15.02	13.71	0.87	0.71
17	1.15	1.30	15.33	14.30	0.35	0.23
18	1.53	1.77	16.42	15.72	0.63	0.46
19	1.08	1.12	16.72	15.81	0.69	0.50
20	0.88	0.97	16.98	16.02	0.33	0.22
21	1.84	1.94	17.60	16.92	0.40	0.26
22	1.41	1.66	18.34	17.62	0.13	0.09
23	0.91	1.03	18.67	17.85	0.24	0.17
24	1.20	1.34	20.14	18.99	0.63	0.75
25	2.18	2.36	21.63	19.24	0.25	0.20
26	1.82	1.90	14.11	13.60	0.41	0.36
27	2.20	2.41	14.54	13.23	1.55	1.32
28	0.56	0.53	9.42	8.83	0.24	0.19
29	1.34	1.46	18.42	17.66	1.28	1.10
30	2.98	3.32	5.26	4.43	1.72	1.54
Range	0.56–3.01	0.53–3.46	1.81–21.63	1.61–19.24	0.10–2.19	0.08–2.01
Mean	1.45 (±0.112) *	1.59 (±0.127)	12.65 (±0.967)	11.74 (±0.950)	0.65 (±0.101)	0.57 (±0.093)

*Figures in parenthesis indicate the standard error of mean

Bablok (1983) were obtained for data sets of each parameter extracted with proposed and reference extractant (Figure 1). CUSUM a sequential analysis technique (cumulative sum) for linearity was used to evaluate how well a linear model fits the data.

Passing & Bablok's regression model validation by CUSUM *test results and interpretation:*

Nicotine

(1) Passing and Bablok's regression (n = 30) of nicotine in percent estimated by the method using test extractant (x) on the method using reference extractant (y) was

$$y=0.044+0.8876\ x$$

(2) Linear model validity by CUSUM test for linearity shows there was no significant deviation from linearity (P = .63).

Reducing sugars:

(1) Passing and Bablok's regression (n = 30) of reducing sugars in percent estimated by method using test extractant (x) on method using reference extractant (y) was

$$y = 0.594+1.0214\ x$$

(2) Linear model validity by CUSUM test for linearity shows there was no significant deviation from linearity (P = .63).

Chlorides:

(1) Passing and Bablok's regression (n = 30) model of chlorides in percent estimated by method using test extractant (x) on method using reference extractant (y) was

$$y = 0.0572+1.131\ x$$

(2) Linear model validity by CUSUM test for linearity shows there was no significant deviation from linearity (P = .91).

The residual standard deviation (RSD) was 0.039, 0.397, and 0.055% for nicotine, reducing sugars, and chlorides, respectively (Table 1a & b). The residual standard deviation (RSD) is a measure of the random differences between the two methods. 95% of random differences are expected to lie in the interval ± 1.96 RSD. If this interval is large, the two methods may not be in agreement. The residual standard deviation (RSD) was measured for two extractants in the study and found 95% of random differences were within the interval indicating both are in agreement (Table 1).

Spearman's rank correlation coefficient (ρ) with P-value at 95% confidence interval was also obtained to know the relationship between two for the three analytes measured (Table 1b). It showed good and high correlation between the extractants for data on nicotine (0.993), reducing sugars (0.982), and chlorides (0.972).

Youden graphical plot test: The data were further examined with Youden plot method (Youden 1959) to visualize the analyzed data of nicotine, reducing sugars, and chlorides. The rectangles in the graph representing 1SD, 2 SD, or 3 SD on both the x-axis and y-axis. The distribution of data points near to diagonal reference line and wild points were far more less indicating values of the proposed extractant to values of the reference extractant were better compared and within the limits of deviation (Figure 2a, 2b & 2c).

Table 1b. Passing and Bablok's regression and Spearman rank correlation (n = 30) statistics of comparison of values of test variables from proposed (A) and reference (B) extractants.

Leaf quality parameters	Nicotine (%)		Reducing sugars (%)		Chlorides (%)	
Statistics	(A)	(B)	(A)	(B)	(A)	(B)
Lowest value	0.53	0.56	1.610	1.810	0.08	0.10
Highest value	3.46	3.01	19.24	21.63	2.01	2.19
Arithmetic mean	1.59	1.45	11.74	12.65	0.56	0.67
Standard deviation	0.69	0.61	5.21	5.30	0.51	0.56
Standard error of the mean	0.127	0.112	0.950	0.967	0.093	0.101
RSD	0.039		0.397		0.055	
±1.96 RSD interval	−0.076 to 0.076		−0.853 to 0.853		−0.129 to 0.129	
Spearman rank correlation coefficient (ρ)	0.993 (P < .0001)		0.982 (P < .0001)		0.972 (P < .0001)	

Note: Proposed extractant (A): 10% acetic acid + 4 cc activated charcoal suspension; Reference extractant (B): 20% Methanol, 5% acetic acid, and water (4:1:15) + 2 cc activated charcoal suspension.

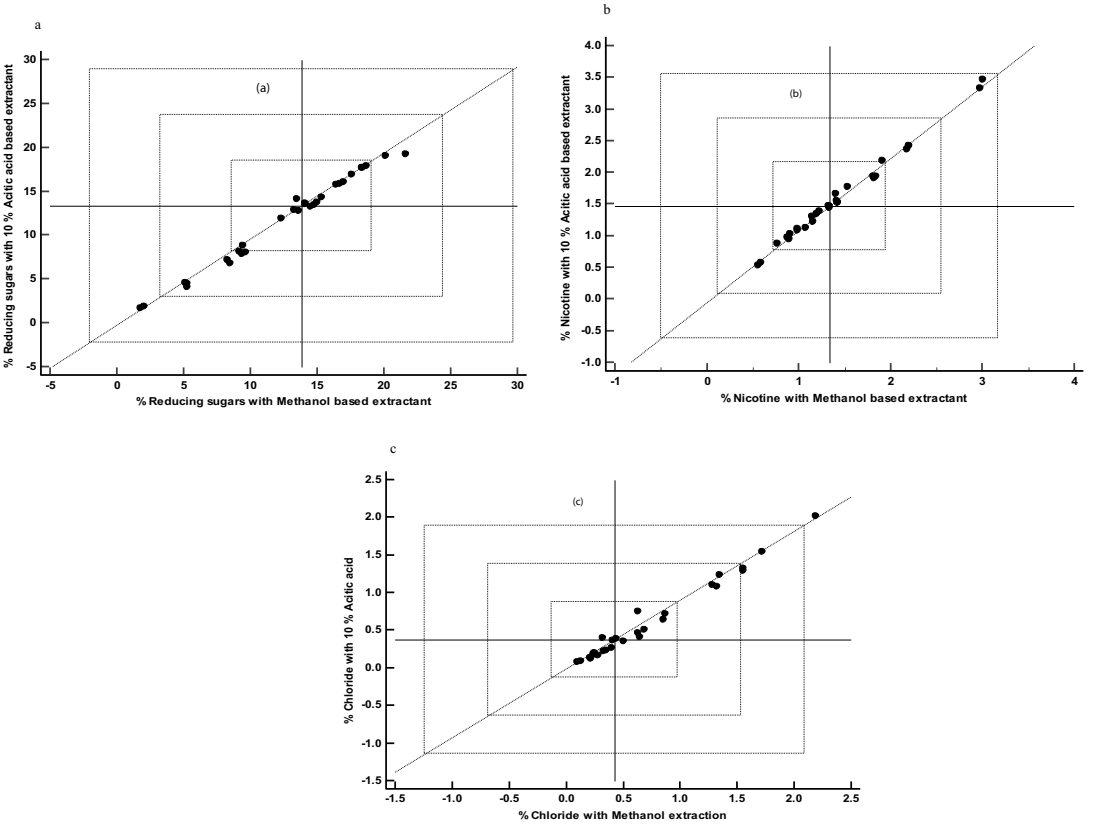

Figure 2. Yoden plots of reducing sugars (a), nicotine (b) and chlorides (c) in tobacco leaf samples estimated by proposed and reference extractant (n = 30).

Cost economics

Estimated cost of assaying nicotine, reducing sugars, and chlorides in 100 cured leaf samples of tobacco by the reference extractant was higher (INR ₹ 445/ 6.09 USD) compared to the cost of analyzing with the proposed extractant (10% acetic acid) was INR ₹ 250//3.42 USD.

Conclusion

The study focused on developing a simple, inexpensive, and less-hazardous extractant for estimation of nicotine, reducing sugars, and chlorides in tobacco cured leaf. Hence, acetic acid at 10% concentration as an extractant was evaluated against 20% methanol-based extractant. The results analyzed through statistical method comparison and validation techniques showed that the values of nicotine, reducing sugars, and chlorides obtained by the proposed extractant had a good correlation and were well compared statistically with their corresponding values obtained by the reference extractant. Thus, 10% acetic acid extractant could be used as an alternative to the hazardous methanol-based extractant in concurrent estimation of nicotine, reducing sugars, and chlorides present in cured tobacco leaf.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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