Supporting Information for:

A Paper-Based Colorimetric Spot Test for the Identification of Adulterated Whiskeys

Thiago M. G. Cardoso,[†‡] Robert B. Channon,[‡] Jaclyn A. Adkins,[‡] Márcio Talhavini,[§] Wendell K. T. Coltro[†] and Charles S. Henry[‡*]

Affiliations

[†]Instituto de Química, Universidade Federal de Goiás, Goiânia, GO, 74690-900 (Brazil)
[‡]Chemistry Department, Colorado State University, Fort Collins, CO, 80523 (USA)
§Instituto Nacional de Criminalística, Departamento de Polícia Federal, Brasília, DF, 70610-200 (Brazil)

*Corresponding author: chuck.henry@colostate.edu

In this Electronic Supporting Information (ESI): device characterization, solution utilization details, resulting caramel calibration curves for different whiskey brands, and comparison with an official method are presented. Calibration detection of caramel spiked whiskeys were analyzed using different color channels as a more sensitive detection method.

Paper-based Spot Test Fabrication

Paper-based analytical devices (PADs) were fabricated using a previously described procedure.[1] In short; µPAD designs were drawn using Corel Draw™ graphical software (Ottawa, ON, Canada), to generate a spot test layout i.e. a circle with internal and external diameters of 10 and 11.75 mm respectively. The devices were printed on Whatman qualitative paper #1 (Little Chalfont, United Kingdom) using a Xerox wax printer (model ColorQube 8870, Rochester, Nova York, United States of America). The printed device was placed on a pre-heated hot plate at 110 ºC for 2 min, to permeate the wax from the paper surface through the paper to generate hydrophobic wax barriers. The back-side of the paper was then laminated at 120 ºC using a thermal laminating pouch (Scotch™, St. Paul, Minnesota, United States of America) with 0.25 mm thickness. This left the top of the spot tests open for solution addition within the laminated bottom and wax printed barriers of the small well devices.

Modification of Paper Wells for Caramel Color Detection

A five-step process was used to modify each spot test for caramel color detection: 5 µL of 0.5 mol L^{-1} citric acid (Fisher Scientific), 10 µL aliquot of sample or standard solution, 5 µL of 1.25 mol L^{-1} NaOH (Sigma-Aldrich), and 5 µL of 0.2 mol L^{-1} sodium phosphate buffered saline (PBS, pH 6.0) (Sigma-Aldrich) were added sequentially and left to dry for 10 min between each addition. Finally, 10 µL of a solution containing detection enzymes and chromogenic reagents was added and left to dry for 30 min. This solution comprised of a 1:1 ratio of 120 U mL^{-1} glucose oxidase from *Aspergillus niger* (147.9 U mg^{-1}) (Sigma-Aldrich) and 30 U mL^{-1} peroxidase from *horseradish* (59 U mg^{-1}) (Sigma-Aldrich), prepared in 0.1 mol L^{-1} PBS (pH 6.0), 4 mmol L^{-1} 2- 4-aminoantipyrine (4-AAP) (Sigma-Aldrich) and 8 mmol L^{-1} sodium 3,5-dichloro-2-hydroxybenzesulfonate (DHBS) (Sigma-Aldrich).

Colorimetric Detection

After drying, the spot tests were imaged for colorimetric detection using a Xerox scanner (model Documate 3220, Rochester, Nova York, United States of America) with 600 dpi resolution and saved in TIF formatting. The color intensity of each image was analyzed in Corel Photo-Paint™ (Ottawa, ON, Canada), using a histogram software tool to measure pixel intensity. Detection within each well was conducted using a circular mask with the same internal diameter of the spot test to ensure repeatable and consistent measurements. For the colorimetric analysis in the RGB color channel, the pixel intensity was directly extracted from the digitalized image without any previous conversion. On the other hand, the analysis in the CMYK, magenta and yellow color channels was performed after converting the digitalized image from 24 to 32 bits. This conversion allowed the colorimetric analysis of an image previously digitalized in the RGB color space in the CMYK scale as well as its individual color channels.

Principal Component Analysis (PCA)

Principal component analysis (PCA) was performed through Microcal™ Origin™ version 9 software (Northampton, MA, USA). The pixel intensity values of each sample in the different color channels (RGB, CMYK, magenta and yellow) were extracted from Corel Photo-Paint™ software and analyzed by PCA. As well-defined in the textbook authored by Miller and Miller[2], PCA is a powerful chemometric tool for reducing the data amount based on orthogonal transformation. Basically, the entire information extracted from all color channels (variables) was analyzed together and the result was expressed as a function of the principal components, which mean uncorrelated variables. The PCA results are often expressed in terms of the score of each principal component generated by a correlation matrix. In our study, 99.86% of the total variance were correlated by two principal components (PC1 and PC2), as displayed in Figure 3.

Based on our view, PC1 and PC2 are probably associated with the concentration of caramel/sucrose and glucose and the brown color of whiskey samples, respectively. The conjunction of PADs, colorimetric detection and PCA generated a quite attractive tool for screening the authenticity of whiskey samples without use analytical curves, i.e., comparing simultaneously the pixel intensity values extracted in different color channels for seized and original samples.

Preparation and Analysis of Mimicked and Adulterated Whiskey Solutions – Generation of Calibration of Curves

A stock solution of mimicked whiskey was prepared by adding 1 mL of commercial caramel color in 100 mL of 40% (v/v) ethanol. Afterwards, a series of eleven solutions were made by dilution ranging from 1000 to 6000-fold. All solutions were diluted with 40% (v/v) ethanol so that only the caramel color concentration changed. Then, 100-μL aliquots of each diluted solution or original whiskey were added to spot tests and allowed to completely dry at room temperature for 1h, before imaging as previously described. The color intensities of the mimicked whiskey solutions were recorded at gray scale, RGB and CMYK channels. Mimicked whiskey solution dilutions were compared to original whiskey samples and a concentration of 667 μL/L commercial caramel color diluted in 40% ethanol (v/v) provided similar color intensities to original whiskey samples. This mimicked whiskey solution (667 μL/L commercial caramel color) was then used to adulterate six original whiskey samples in increasing ratios as follows: 0, 7.5, 22.5, 37.5, 52.5, 67.5, 82.5, and 97.5 % (v/v) of mimicked whiskey diluted into original whiskey. Caramel color detection was performed as described above and color formation results are plotted in Figures S1-3 for RGB, CMYK, and magenta color channels, respectively.

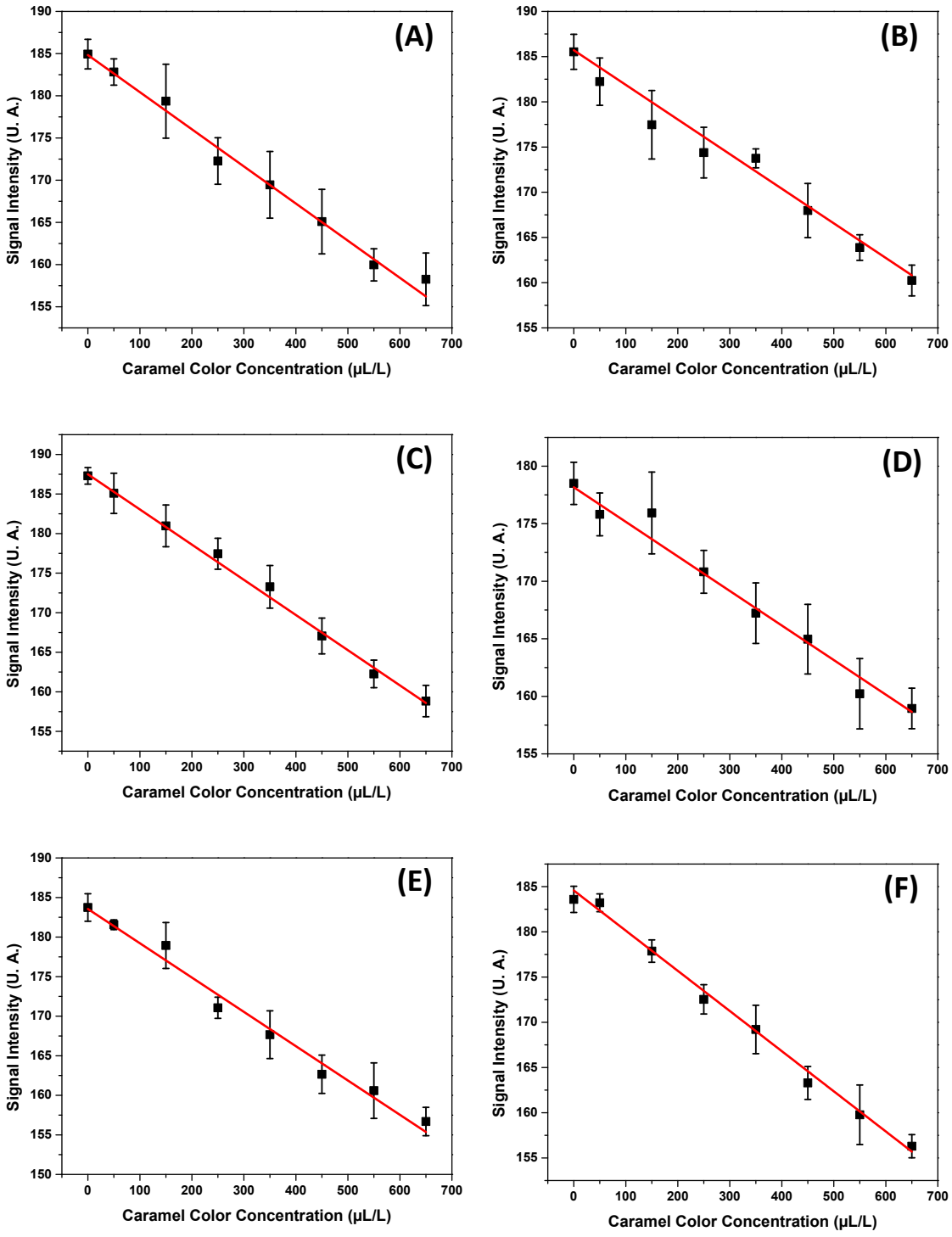

Fig. S1: Analytical curves for caramel detection in whiskey samples using RGB channel. (A) Jack Daniels, (B) White Horse, (C) Ballantines, (D) Black Label, (E) Red Label and (F) Chivas Regal.

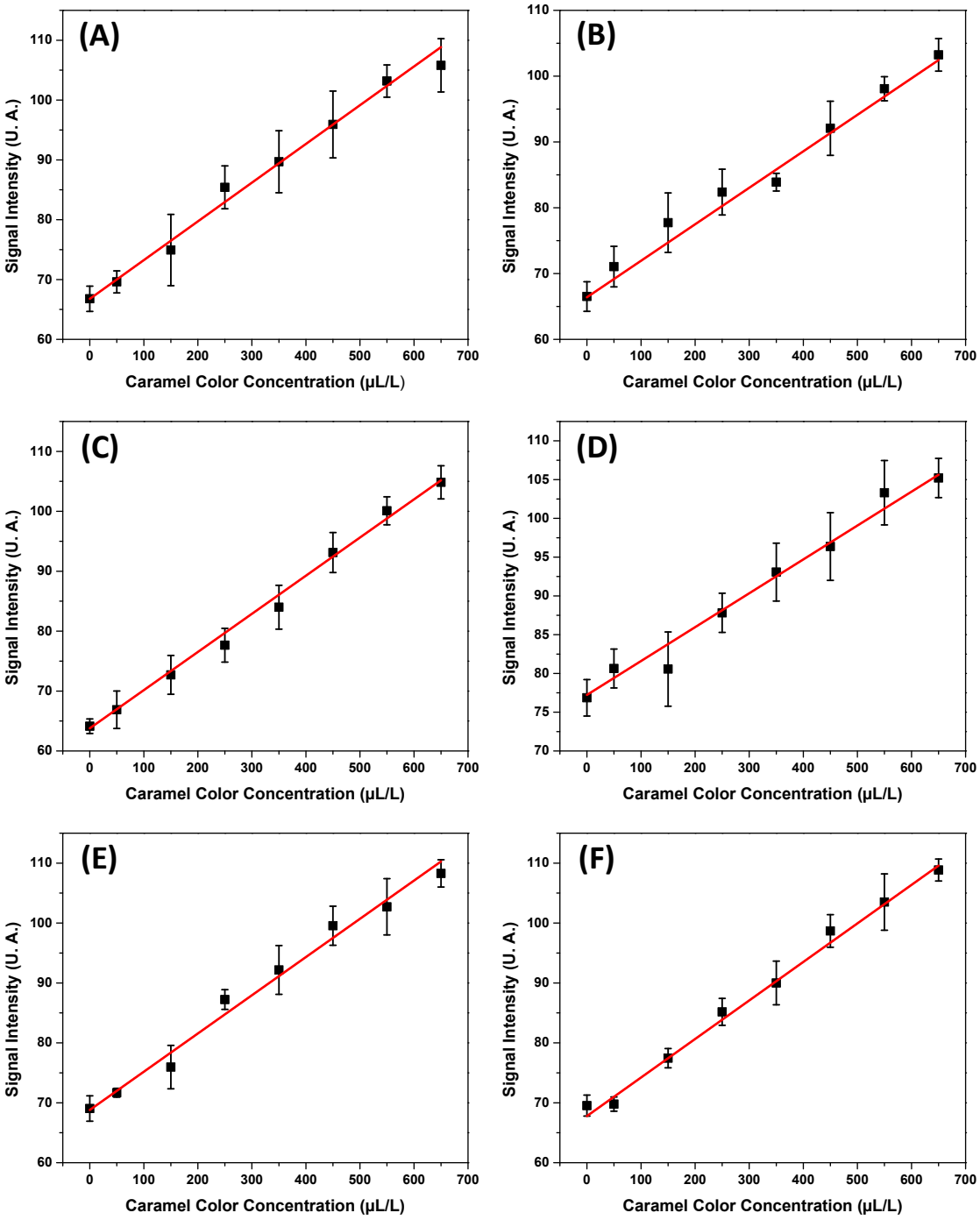

Fig. S2: Analytical curves for caramel detection in whiskey samples using CMYK channel. (A) Jack Daniels, (B) White Horse, (C) Ballantines, (D) Black Label, (E) Red Label and (F) Chivas Regal.

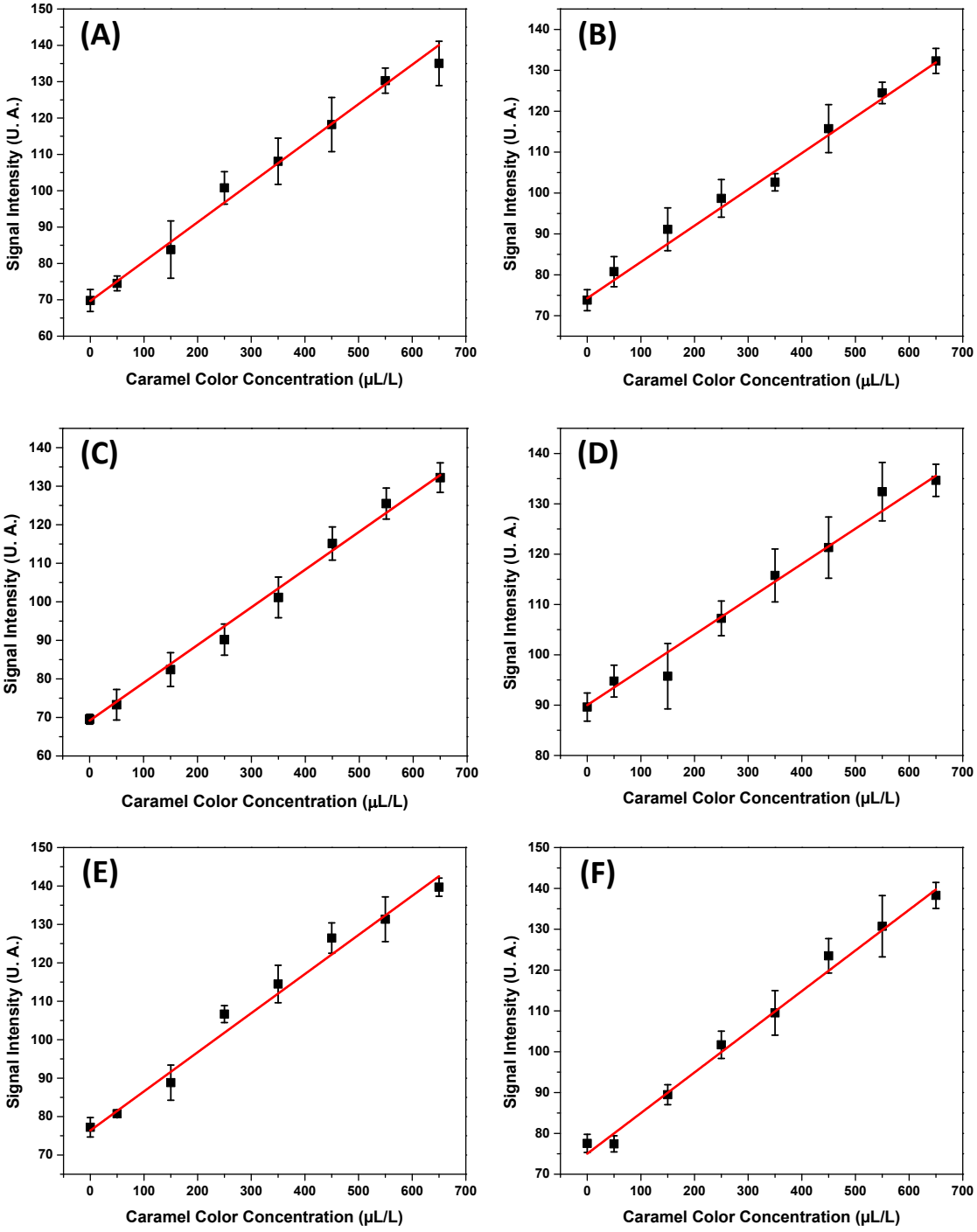

Fig. S3: Analytical curves for caramel detection in whiskey samples using Magenta channel. (A) Jack Daniels, (B) White Horse, (C) Ballantines, (D) Black Label, (E) Red Label and (F) Chivas Regal.

Identification of Seized Whiskey Samples

The color intensity of different seized whiskey samples was measured according to the following procedure: First, a 100-µL aliquot of whiskey sample was added to a spot test and allowed to completely dry at room temperature for 1h. Then, color intensity was measured using the yellow color channel in comparison to a sample of original whiskey of that brand. This protocol was used to analyze 47 seized whiskey samples claiming to be from 4 different brands (Red Label, Black Label, White Horse, and Ballantines), which were supplied by the Brazilian Federal Police. The color intensity of seized whiskey samples was then compared to the recorded profile for the unadulterated sample profile of the claimed brand.

Analysis of Total Sugar Concentration Levels

To demonstrate the reliability of the proposed PADs, the total sugar concentration was determined through a volumetric method, which is the official approach implemented and validated by national laboratories of the Ministry of Agriculture, Livestock, and Supply to verify the authenticity of alcoholic beverages in Brazil.

The official method is based on the titration of reducing sugars with copper (II) ion in alkaline solution and heating.[3] In the reaction, Cu(II) is reduced to Cu(I) changing from blue to brick red, indicating the formation of Cu_2O. Due to its routine application in the beverages analysis as an official technique approved by the National Institute of Metrology, Quality and Technology from the Brazilian Government, this method was selected to perform a side by side comparison with the results achieved by using paper-based analytical devices. The sample analysis was performed by the Physical-Chemical Analysis Laboratory of Beverages and Vinegars from LANAGRO-GO (Goiânia, GO, Brazil). The detection and quantification limits of the volumetric method for the analysis of total sugar are 0.13 and 0.60 g/L, respectively.

For a side-by-side comparison, five seized samples of two brands (Red Label and White Horse) were analyzed by both official method and our proposed PAD approach. The achieved results are displayed in Table S1 below. All seized samples from the Red Label whiskey revealed the presence of a strong magenta coloration inside the spot test, suggesting an adulterated whiskey. Importantly, the intense coloration suggests high concentration of total sugar, which was confirmed by the official method with concentration levels ranging from 0.96 to 1.44 g/L. For the White Horse whiskeys, PADs were able to identify the presence of caramel/sucrose and glucose in all five samples. One of them (Sample #4) exhibited very strong magenta coloration with pixel intensity near the color channel saturation. For this sample, the analysis through the official method provided high concentration of sugars (11.46 g/L). On the other hand, the sugar concentration levels for the other four samples (#1, #2, #3 and #5) were below the limit of quantification (LOQ). For both samples, the analysis of original whiskeys (Red Label and White Horse) indicated small amount of sugar (see weak magenta coloration inside spot tests) by PADs, which was then confirmed by the official method (where the concentration levels were below the limit of detection (LOD)).

According to the presented data, it can be inferred that the use of PADs has a great potential to provide a reliable screening of the sample authenticity. The side-by-side comparison with an official method suggests that PADs can be able to detect adulterations below of the LOQ provided by the official method.

Table S1. Comparison of the sample analysis through paper-based analytical methods and official method according to the Brazilian regulation to verify the authenticity of seized whiskey samples.

Red Label				White Horse			
Samples	***PADs***	***Pixels Intensity (A.U.)***	***Official Method* (g/L)***	***Samples***	***PADs***	***Pixels Intensity (A.U.)***	***Official Method (g/L)***
Original		68.7±7.5	< LOD	**Original**		69.0±3.7	< LOD
#1		200.2±8.2	1.2	**#1**		133.1±8.2	< LOQ
#2		166.1±7.7	0.96	**#2**		130.6±3.7	< LOQ
#3		169.4±9.4	0.96	**#3**		124.2±10.1	< LOQ
#4		191.9±26.9	1.25	**#4**		224.1±1.8	11.46
#5		220.6±6.4	1.44	**#5**		124.0±7.4	< LOQ

References

1. E. Carrilho, A. W. Martinez and G. M. Whitesides, *Anal. Chem.*, 2009, **81**, 7091-7095

2. Miller, J. N.; Miller J. C.; Statistics and Chemometrics for Analytical Chemistry, 6th ed., Pearson: Harlow, 2010.

3. BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. Secretaria de Defesa Agropecuária. Instrução Normativa nº24, de 08 de setembro de 2005. Aprova o Manual Operacional de Bebidas e Vinagres. **Diário Oficial da União**, DF, 20 set. 2005. Seção 1, p. 11.