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Determination of Limonene Chirality in Oils Obtained from Different Types of Citrus Waste Peels in Türkiye

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Abstract: Limonene constitutes a significant amount in citrus oils. It has a chiral structure and has two different optically active isomers, R-limonene and S-limonene, which are symmetrical to each other. Determining the chiral configurations of limonene plays an important role in determining the beneficial use areas of essential oils. Citrus oils are used in a wide variety of industrial areas, depending on their limonene content. This paper presents the analytical method optimization, validation, and chirality studies of limonene in the citrus oils acquired from different citrus waste peels in Türkiye. An inlet temperature of 250 °C and an injection volume of 2 µL were decided as the optimal conditions for the most accurate measurement of both limonenes in the citrus oil. This method produced results for linearity, sensitivity (LODs and LOQs), repeatability, and reproducibility that were acceptable within the scope of the validation studies. The chirality of limonene was investigated in twenty-six citrus oils (fifteen orange oils, six lemon oils, four mandarin oils, and one grapefruit oil) in Türkiye. While the content of R-limonene in orange oil varied between 56.39% and 72.85%, the content of S-limonene changed from 2.53% to 5.71%. Whereas the constituent of R-limonene in lemon oils ranged from 54.73% to 73.99%, the content of S-limonene varied between 3.78-4.79%. In mandarin oils, the content of R-limonene was determined to be 58.02% and 65.05%, while the content of S-limonene was found as 3.05% and 4.87%. In single grapefruit oil, R-limonene content was 60.69% and S-limonene content was 3.12%.

Keywords: Chirality, Citrus peel, Essential oil, Gas Chromatography, Mass Spectrometry, Limonene.

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1. INTRODUCTION

Limonene is a naturally occurring compound found in the peels of citrus fruits such as lemons, mandarins, oranges, and grapefruits. It is commonly used as a flavoring agent in food and beverages, as well as a fragrance in cosmetics and cleaning products (1). The limonene molecule ($C_{10}H_{16}$) has a chiral structure and has two enantiomers, which are mirror images of each other (Figure 1). R-limonene (or D) and S-Limonene (or L), which are symmetrical to each other (2,3). R-limonene is one of the enantiomers of limonene that is the naturally occurring form and has a fresh citrus aroma, often described as orange-like. This enantiomer is commonly found in citrus fruits and is used in various applications, including flavoring agents, fragrances, and cleaning products. S-limonene is a specific enantiomer of limonene, which is a naturally occurring compound found in the peels of citrus fruits. They have the same chemical formula but differ in their spatial arrangement. In terms of its potential beneficial uses, S-limonene shares similar properties and potential health benefits as limonene in general (5,6).

There is a significant amount of scientific research conducted on limonene in citrus oils due to its wide range of applications and potential health benefits. Here are a few examples of scientific research studies related to limonene in citrus oils: (a) "Anticancer activity of citrus peel oil components limonene". The researchers found that these compounds exhibited anti-tumor activity against various types of cancer cells (7-9). (b) "Antioxidant and anti-inflammatory roles of limonene". Limonene's potential therapeutic applications in the prevention and treatment of several diseases are also highlighted (10-13). (c) "Limonene and its role in chronic diseases". Researchers are looking into limonene's potential for use in the treatment and prevention of chronic

illnesses like diabetes, obesity, and cardiovascular diseases. They discuss the mechanisms of action and the potential health benefits of limonene (14). (d) "Antimicrobial activity of the citrus fruit peel oils against pathogenic bacteria". The studies investigated the antimicrobial activity of citrus fruit peel oils, including limonene, against various pathogenic bacteria. The results showed that these oils exhibited significant antimicrobial effects, suggesting their potential use as natural antimicrobial agents (15,16). (e) "Limonene inhalation reduces stress and improves immune function". The researchers found that limonene inhalation reduced stress-induced behaviors and improved immune function, suggesting its potential as a natural stress-relieving agent (17,18).

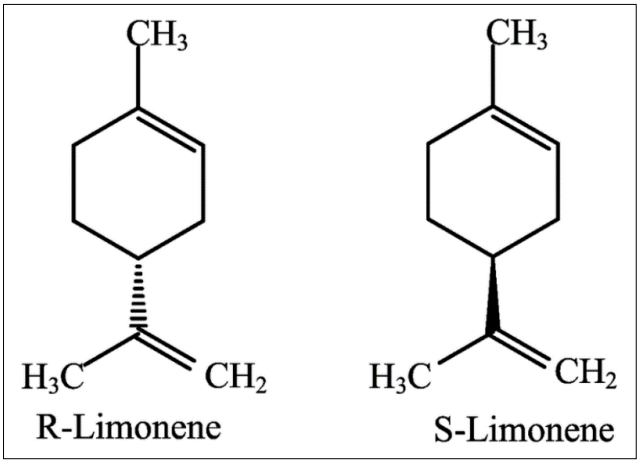

Figure 1: The chemical structure of limonene enantiomers (4).

The detection of limonene in citrus oils can be done using various analytical techniques. Here are a few commonly used methods: (a) Gas Chromatography (GC): GC is a widely used instrumental technique for analyzing volatile compounds like limonene. It involves separating the components of a sample based on their volatility and then detecting and quantifying them. GC coupled with a flame ionization detector (FID) or a mass spectrometer (MS) is commonly used for limonene analysis (19). (b) High-Performance Liquid Chromatography (HPLC): HPLC is another technique used for the analysis of limonene in citrus oils. It involves separating the components of a sample based on their chemical properties using a liquid mobile phase. Detection can be done using UV-Vis spectroscopy or other detectors (20). (c) Fourier Transform Infrared Spectroscopy (FTIR): FTIR spectroscopy can be used to identify and quantify limonene in citrus oils based on the characteristic absorption bands of the compound. It provides information about the functional groups present in the sample (21). (d) Nuclear Magnetic Resonance (NMR) Spectroscopy: NMR spectroscopy can be used to identify and quantify limonene in citrus oils. It provides information about the chemical structure and can be used to differentiate between different isomers of limonene (22). These are just a few examples of the techniques used for the detection of limonene in citrus oils. The choice of method depends on factors such as sensitivity, selectivity, and the equipment available in the laboratory.

In this study, we present the analytical method optimization, validation, and chirality studies of limonene in the citrus oils obtained from different citrus waste peels in Türkiye. It is thought that this study will contribute to the accurate chiral identification of limonene in citrus oils and different plant-based oils. The authors believed that the results of method optimization and validation studies would provide significant support for analytical method studies in this field.

2. EXPERIMENTAL SECTION

2.1. Reagents and Chemicals

Single standards (≥99.9% purity) of S-limonene (1000 µg/mL in isopropanol) and R-limonene (1000 µg/mL in isopropanol) in ampoule form were purchased from Dr. Ehrenstorfer (Augsburg, Germany). To make the corresponding stock solutions, Merck (Darmstadt, Germany) provided hexane with the highest analytical purity (GC gradient grade). By combining the aforementioned standards at equal concentrations (100 and 250 µg/mL stock solutions in hexane), the desired concentrations were formed. The dilution of the required solutions for matrix-matched calibration curves or validation assays in hexane was conducted progressively.

2.2. Analytical Method Optimization

GC is a programmable analytical technique to successfully separate relative analytes in an analysis. Some GC parameters were optimized to get the best MS and analysis conditions in the experiments in this study. Thus, trial runs were carried out on the inlet temperature and injection volume parameters of the GC-MS device to determine the optimal conditions for the sensitive and accurate determination of enantiomers of limonene.

2.3. GC-MS Analytical Condition

Within the scope of this study, the determination of chirality of limonene (the amounts of R-limonene and S-limonene) was done by using GC-MS with Cyclosil-B column (length 30 m, id. 0.250 mm, film thickness 0.250 µm; Agilent Technologies) as chiral column. Analytical separation was carried out by

temperature-programmed analysis and detection by electron ionization MS in full-scan mode. Details of the GC analysis program for chirality testing of limonene in citrus oils are presented in Table 1.

Table 1: GC analysis program for chirality testing of limonene in citrus oils.

Instrument	Agilent 6890N GC Single Quadropole MS		
Carrier Gas	Helium		
Carrier Gas Flow	1.1 mL/min.		
Column	Cyclosil-B		
Column Length	30 m		
Column Inner Diameter	0.25 mm		
Film Thickness	0.25 µm		
Detector Type	MS		
Detector Temperature	220 °C		
Electron Energy	70 eV		
Injection Volume	2 µL		
Auxiliary Temperature	230°C		
Oven Temperature Program	**Ratio (°C/min)**	**Temperature (°C)**	**Retention Time (min.)**
Initial		50	3
	10	100	5
	20	220	5

2.4. Sample Collection and Sample Preparation

The sampling studies were carried out to determine the chirality of limonene content in twenty-six citrus oils (fifteen orange oils (POR), six lemon oils (LİM), four mandarin oils (MAN), and one grapefruit oil (GREY)) from Anadolu Etap Agriculture and Food Products Industry and Trade Inc. obtained from Türkiye at different times. Citrus peels were used in the cold-pressed extraction process to produce the citrus oils that were provided. The TÜBITAK MAM laboratories received these oils in tightly sealed 1L containers with a cold chain. Up until the end of the studies, all samples were kept at +4 °C.

The water content in the citrus oil samples was eliminated by passing them through a sodium sulfate column before GC/MS analysis was performed on them. About 0.5 g of citrus oil samples were added into a glass tube and the samples were then spiked with 4.5 mL of hexane. In an ultrasonic bath, they were mixed for ten minutes. Finally, 2 µL of the sample in hexane was injected into the GC-MS after the mixture was diluted to 1 mL and put into a vial.

2.5. Performance of GC-MS method

The validation of the optimized GC-MS method involved conducting various studies, including an assessment of parameters such as linearity, sensitivity (limit of detections (LODs) and the limit of quantifications (LOQs)), repeatability, and reproducibility. This validation process adhered with EURACHEM Guidelines (23) and the Guidelines for Standard Method Performance Requirements (24).

3. RESULTS AND DISCUSSION

3.1. Assessment of Analytical Method Performance Studies

In this study, the retention times of R-limonene and S-limonene were determined in the GC chromatogram, at first. Figure 2 shows the GC-MS chromatogram obtained from the analysis of a mixture of R-limonene and S-limonene at a concentration of 10.0 mg/L. The retention times (min.) of S-limonene and R-limonene were 8.07 and 8.23, respectively.

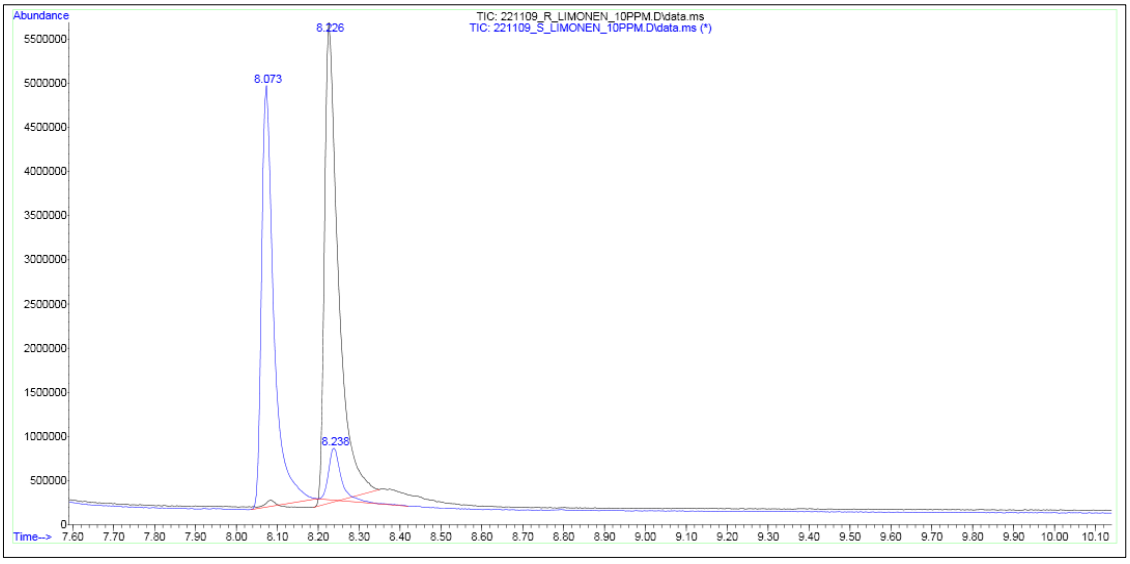

Figure 2: GC-MS chromatogram of R-limonene and S-limonene.

The method optimization stage aims to find the most suitable conditions for the most successful chromatographic separation of both limonenes. Considering the volatility of limonene, the determination of optimum conditions of the analytical method was conducted depending on the parameters of inlet temperature and injection volume of the GC-MS. First, the behaviour of terpenes was studied at five different inlet temperatures (230 °C, 240 °C, 250 °C, 260 °C, and 270 °C) (Figure 3a). After determining the ideal inlet temperature, the signal efficiency of the limonene on the GC system was assessed at four different injection volumes (0.5 µL, 1 µL, 1.5 µL, and 2 µL) (Figure 3b). In accordance with the graphical results in Figure 3, an inlet temperature of 250 °C and an injection volume of 2 µL were decided as the optimal conditions for the most accurate measurement of both limonenes in the citrus oil.

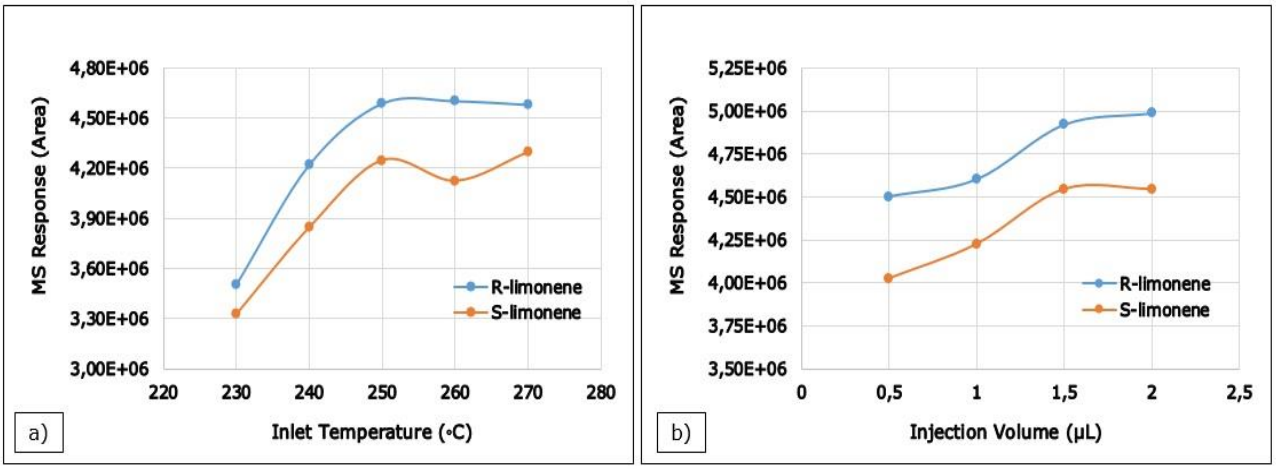

Figure 3: Effect of a) inlet temperature (°C), and b) injection volume (µL) on MS measurement of R-limonene and S-limonene.

Table 2 demonstrates information about the analytical method performance results of limonene enantiomers in citrus oils. Seven different concentration levels of each limonene - 1.00 mg/L, 2.00 mg/L, 5.00 mg/L, 10.0 mg/L, 25.0 mg/L, 50.0 mg/L, and 100 mg/L - were used to form the calibration curves for each one (Figure 4). The linear dynamic range of measurements for both was from 1.00 mg/L to 100 mg/L. As seen in Figure 4, the calibration coefficients (R^2) of S-limonene and R-limonene were 0.9991 and 0.9976, respectively. In accordance with other comparable method validation studies in the literature (25-30), the correlation coefficient of a calibration curve denotes a good linear regression if it is greater than 0.99. In this regard, the values (R^2) of S-limonene and R-limonene indicate that the relevant calibration graphs have good linearity for accurate measurement.

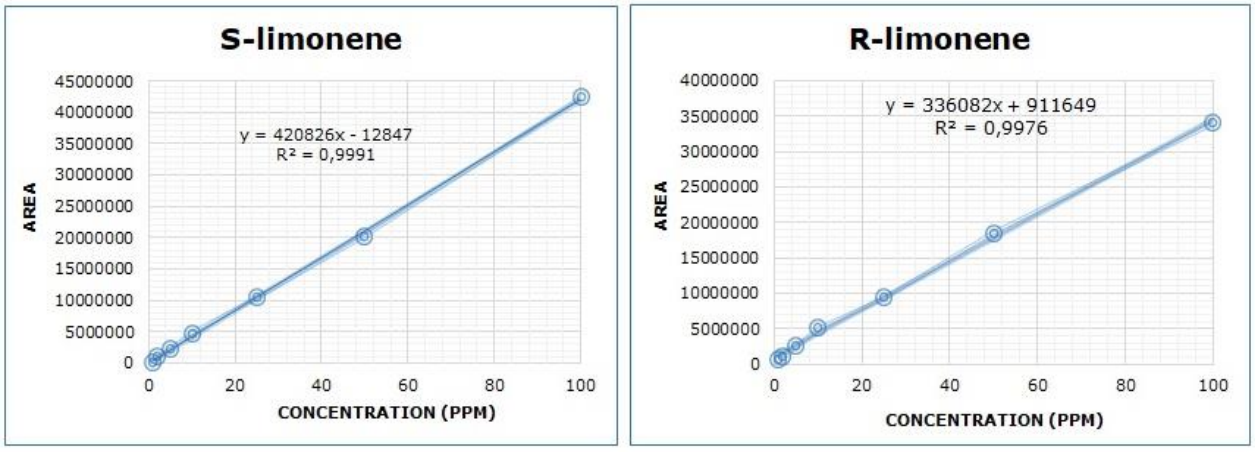

Figure 4: The linearity of calibration graphs of R-limonene and S-limonene.

To determine the LOD and LOQ of both limonenes, six injections of the mixture of relevant standards at different concentrations (1.00 mg/L and 2.00 mg/L) were made. The LODs were calculated taking into consideration the limonene peaks, which are clearly visible over the background noise in the chromatogram of the GC system. The LOD and LOQ values of both limonenes correspond to the signal-to-noise ratio multiplied by 3 and 10. The LODs of R-limonene and S-limonene were found to be 0.08 mg/L and 0.09 mg/L, while their LOQs were calculated as 0.24 mg/L and 0.26 mg/L, respectively. As seen in Table 2, the values of LODs and LOQs of both limonenes are at a level comparable to studies in the literature in this field (31-33).

Table 2: Analytical method performance results of limonene enantiomers in citrus oils.

Parameters			R-limonene	S-limonene
Linear dynamic range (mg/L)			1-100	1-100
LOD (mg/L)			0.08	0.09
LOQ (mg/L)			0.24	0.26
Accuracy (5 mg/L)		Recovery (%)	90.82	4.86
		RSD (%)	2.21	5.28
Repeatability (10 mg/L; n=6)		Mean Conc. (%)	8.86 ± 0.31	9.00 ± 0.26
		Recovery (%)	88.62	89.98
		RSD (%)	3.58	2.98
(Reproducibility (5 mg/L; n=6)	Day-1	Mean Conc. (%)	4.40 ± 0.05	4.55 ± 0.10
		Recovery (%)	88.02	91.09
		RSD (%)	1.07	2.11
	Day-2	Mean Conc. (%)	4.56 ± 0.07	4.48 ± 0.10
		Recovery (%)	91.28	89.53
		RSD (%)	1.54	2.24

RSD: Relative Standard Deviation

The accuracy test involved injecting a standard solution with a concentration of 5 mg/L into the mixture of citrus oils and taking six measurements to calculate the recovery of each limonene. The accuracy of the optimized method was required to be between 70 and 120% (accepted recovery range) (34, 35). Table 3 indicates that the mean recovery of R-limonene and S-limonene was 88.62% and 89.92% with an RSD of 2.21% and 5.28%, respectively. These results are within the acceptance range specified above, indicating that the method is sufficient in terms of measurement accuracy.

Six consecutive measurements at a concentration of 10.0 mg/L of each limonene were used to determine the precision's repeatability. Six consecutive measurements at a concentration of 5.00 mg/L on two different days were used to actualize the reproducibility of one person. In the precision test, it is considered acceptable if the results of repeatability and reproducibility are 15% lower in terms of RSD (34, 36). As shown in Table 2, the RSDs of both limonenes in the repeatability test were between 3.58% and 2.98%. In the reproducibility test, the RSDs of R-limonene changed from 1.07% to 1.54%, whereas the RSDs of S-limonene varied from 2.11% to 2.24%. The optimized method possesses a satisfactory precision compared to the other research in the literature (34, 36-38).

3.2. Determination of Chirality of Limonene in Citrus Oils

The chirality of limonene in citrus oils made from various citrus waste peels was ascertained using the optimized and validated GC-MS method. The details of the chirality test results of limonene in different types of citrus oils in Türkiye by GC-MS are presented in Table 3. The results for total limonene content in relevant citrus oils were taken from previous work (19). R-limonene and S-limonene contents were calculated proportionally based on these results. The chirality of limonene was investigated in twenty-six citrus oils in Türkiye. While the content of R-limonene in orange oil varied between 56.39% and 72.85%, the content of S-limonene changed from 2.53% to 5.71%. Whereas the content of R-limonene in lemon oils ranged from 54.73% to 73.99%, the content of S-limonene varied between 3.78-4.79%. In mandarin oils, the content of R-limonene was determined to be 58.02% and 65.05%, while the content of S-limonene was found to be 3.05% and 4.87%. In single grapefruit oil, R-limonene content was 60.69% and S-limonene content was 3.12%.

Table 3: Chirality test results of limonene in different citrus oils in Türkiye.

Type of Citrus Oil / Ingredient	Total Limonene (%)	R-limonene (%)	S- limonene (%)
LIM-1	62.54	57.75	4.79
LIM-2	58.56	54.73	3.83
LIM-3	62.29	58.51	3.78
LİM-4	69.92	65.94	3.98
LİM-5	74.67	70.55	4.12
LİM-6	78.08	73.99	4.09
MAN-1	62.89	58.02	4.87
MAN-2	66.31	63.26	3.05
MAN-3	69.91	65.05	4.86
MAN-4	67.82	63.05	4.77
GREY-1	63.81	60.69	3.12
POR-1	70.48	67.09	3.39
POR-2	66.88	61.69	5.19
POR-3	68.25	62.70	5.55
POR-4	70.32	65.98	4.34
POR-5	60.49	56.39	4.10
POR-6	66.14	62.79	3.35
POR-7	74.27	68.56	5.71
POR-8	73.17	69.22	3.95
POR-9	71.31	66.95	4.36
POR-10	75.12	72.59	2.53
POR-11	67.67	63.41	4.26
POR-12	66.87	61.46	5.41
POR-13	67.58	63.09	4.49
POR-14	65.36	62.11	3.25
POR-15	78.06	72.85	5.21

4. CONCLUSION

This paper presented detailed information about the analytical method optimization, validation, and chirality studies of limonene in the citrus oils obtained from different citrus waste peels in Türkiye. An analytical method containing a chiral column (Cyclosil-B) in the GC–MS was optimized for the simultaneous determination of R-limonene and S-limonene in citrus oils (orange oil, lemon oil, mandarin oil, and grapefruit oil) at first. Then, the validation of the optimized GC-MS method was performed with some studies in accordance with the related international guidelines. Finally, the amount of both limonenes in citrus oils was measured with the optimized and validated GC-MS method. At the end of the determination of optimal conditions, an inlet temperature of 250 °C and an injection volume of 2 µL were determined as the optimal conditions in the measurement of both limonenes in the citrus oil, precisely. This method produced the results for linearity, sensitivity (LODs and LOQs), repeatability, and reproducibility that were desirable. In Türkiye, while the content of R-limonene in orange oil varied between 56.39% and 72.85%. the content of S-limonene changed from 2.53% to 5.71%. Whereas the content of R-limonene in lemon oils ranged from 54.73% to 73.99%. the content of S-limonene varied between 3.78-4.79%. In mandarin oils, the content of R-limonene was determined as 58.02% and 65.05%, while the content of S-limonene was found as 3.05% and 4.87%. In single grapefruit oil, R-limonene content was 60.69% and S-limonene content was 3.12%. The study provides a significant alternative method for the sensitive, accurate, and simultaneous identification of limonene in citrus oils and different plant-based oils in terms of literature. With the analytical method's optimum conditions, it will prevent analytical devices from losing performance in a shorter time by providing significant savings in energy and time.

5. CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

6. ACKNOWLEDGMENTS

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