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An HPLC/CAD method for determination of fatty acids in metered dose inhalation products: a candidate leachable test method

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Abstract: There is no universal method and validation parameters for leachable studies for potential impurities in a metered-dose inhalation drug in the pharmaceutical industry, and this study aims to develop a new method to determine the leachability analysis of some fatty acids (myristic, linoleic, palmitic, oleic, elaidic, stearic, and arachidic acids) by HPLC/CAD that may leach from the valve portion of the product as potential impurities in metered-dose inhalers. The method was found suitable for leachability analysis of fatty acids in metered dose inhalation medication by HPLC/CAD by changing the method parameters, instrument, and detector settings and validated by determining sensitivity, linearity, range, specificity, accuracy, and recovery. In the developed analytical method, the C18 column proved to be an effective stationary phase for chromatographic separation. The mobile phase was determined to be a gradient system, comprising mobile phase A (0.5 mL of formic acid in 1000.0 mL of pure water) and mobile phase B (0.5 mL of formic acid in 1000.0 mL of acetonitrile). The standard solution was stable for 48 hours at 10°C and the sample solution was stable for 3 hours at 10°C.

Keywords: Metered dose inhalation; leachable; fatty acids; high performance liquid chromatography; charged aerosol detector; validation.

1. Introduction

Pharmaceutical products are designed to benefit patients. Ideally, these products should be chemically pure. However, certain impurities may arise during the production and storage of preparations. These impurities do not benefit the patient and may even pose serious risks to their health. It is essential to have knowledge about the quantities properties, and formation effectively controlled or prevented. In this the negative effects caused by impurities will either be completely eliminated or minimized [1-2].

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Leachables are impurities that can migrate from the packaging, packaging system, shipment system, or process equipment directly into the pharmaceutical formulation, potentially affecting the patient. They are also referred to as leachable packaging impurities [3]. Leachables can impact the efficacy, safety, and quality of pharmaceutical products. Therefore, scientifically robust leachables assessment studies are crucial for both manufacturers and suppliers to determine the appropriate conditions for pharmaceutical packaging and shipment systems [4].

Packaging systems consist of all the packaging components that enclose and protect the dosage form. These systems are categorized into primary, secondary, and tertiary packaging systems. Primary packaging components are in direct contact with the product (e.g., an IV bag). Secondary packaging components are in direct contact with the primary packaging component and may provide additional protection for the product (e.g., an overwrap or dust cover for the IV bag). Tertiary packaging components are in direct contact with the secondary packaging component and provide additional protection during shipping or storage (e.g., a shipping carton for an overwrapped IV bag) [5].

Containers and closures are intermediate components that contain pharmaceutical ingredients, excipients, or dosage forms and are in direct contact with the product. Containers (e.g., ampoules, syringes, bottles) and closures (e.g., screw caps, stoppers) are essential packaging components [4].

Leachables management for pharmaceutical products has become a critical part of pharmaceutical development and regulatory submissions, particularly regarding the interaction between the packaging system and the dosage form. The applications are categorized by the route of administration given in the Supporting Information. [4].

A well-planned extractables and leachables study is crucial for assessing the risk of extractables and leachables in the pharmaceutical industry. For this reason, it is essential in the pharmaceutical industry to develop precise and accurate analytical methods that will enable the qualitative and quantitative identification of extractables and leachables components [6].

There is a possibility that some fatty acids may leak as impurities from the valve-containing canister part of our metered dose inhalation product. All fatty acids are organic acids and they are glyceryl esters of carboxylic acids consisting of a long hydrocarbon chain with a methyl group ($-CH_3$) at one end and a water-soluble carboxyl group (-COOH) at the other end [7-8]. The hydrocarbon chains of saturated fatty acids do not contain double bonds; all carbons are saturated with hydrogen. [9-10]. Unsaturated fatty acids contain one or more double bonds in their structures, and their properties vary according to the number of double bonds, the position of the double bonds and their geometric arrangement [9-11].

HPLC was used due to its sensitivity, ease of adaptation for accurate quantitative determinations, suitability for separating non-volatile compounds or structures that can easily degrade with heat, the ability to reuse its column multiple times in a row, and its high reproducibility [5]. The CAD is a detector that can be used in conjunction with HPLC and measures the amount of charged aerosol particles in a sample [12]. It is commonly used in the analysis of compounds that cannot be detected using UV/Vis detectors due to their lack of a chromophore. Additionally, it is preferred because it provides high sensitivity and excellent separation [12-13].

Some studies on both method development and validation using HPLC are available in the literature [14-15]. However, there is no standardized methodology and validation parameters for the conduct of leachability studies, particularly for fatty acids. This makes it particularly important to develop a robust framework for the pharmaceutical industry with this method.

A review of the existing literature identifies several instances of analyses pertaining to N-nitrosamines. Cárdenes et al. developed a new and sensitive method for the quantitative determination of volatile N-nitrosamines in trace amounts using an HPLC-UV system. In their method, they used a C18 column as the stationary phase. For the mobile phase, they performed reverse-phase isocratic separation using a mixture of acetonitrile-USS (55:45, v/v) at a flow rate of 1.2 mL/min. The injection volume was 25 µL, and the fluorescence excitation and emission wavelengths were set to 339 nm and 531 nm, respectively. This developed method was used to study volatile N-nitrosamine derivatives [16].

In the study of Chang *et al.* [17], a feasible and sensitive multi-analyte LC-MS/MS method was used to determine 12 N-nitrosamine derivatives in sartans sold in Taiwan, including the active pharmaceutical ingredients and final products. They operated in positive scan mode using an atmospheric

pressure chemical ionization (APCI) ionization source. In their method, they used a reverse-phase 3 functional group-bound alkyl C18 column as the stationary phase. For the mobile phase, they used ultra-pure water (UPW) solution containing 0.1% formic acid and a solution of acetonitrile/methanol (20:80, v/v) containing 0.1% formic acid. They performed gradient separation with a flow rate of 0.6 mL/min [17].

Since there is no HPLC method for the determination of some fatty acids thought to be leached, this study aimed to develop and validate a new method using High-Performance Liquid Chromatography (HPLC) with a Charged Aerosol Detector (CAD) for myristic, palmitic, stearic, arachidic acids as saturated fatty acids and oleic acid, linoleic acid and elaidic acid as unsaturated fatty acids which can leachable from the valve part of inhalation products with the highest likelihood of packaging component and dosage form interaction.

2. Experimental

2.1. Chemicals and Instruments

An HPLC system (Thermo Scientific Dionex Ultimate 3000) equipped with dual gradient pump, auto sampler, column oven and charged aerosol detector (CAD) was used for analysis. A Poroshell 120 EC-C18 column (150 mm length × 3.0 mm width; 2.7 µm particle size) was used for separation. Chromatographic separation was achieved using timed gradient. The mobile phase consisting of A: 0.5 mL of formic acid into the 1000.0 mL of pure water and B: 0.5 mL of formic acid into the 1000.0 mL of acetonitrile with a flow rate of 1.0 mL/min. The column temperature was 50˚C and sample tray temperature was 10˚C. The injection volume was 20 µL. Diluent was acetonitrile.

The parameters of the CAD detector used are as follows: evaporator temperature nominal: 35.0°C, data acquisition rate:2 [Hz], filter constant:1.0 [s], cad power function:1.00, flow rate:100 pa.

All chemicals used in this study were of analytical reagent grade. As a fatty acid myristic acid (C14:0; 100%, bought from USP, lot number: R14500), linoleic acid (C18:2(9,12); 98%, bought from TRC, lot number: 2-XZL-5-1), palmitic acid (C16:0; 100%, bought from EP, lot number: P0090000/5), oleic acid (C18:1(9); 98.8%, bought from EP, lot number: Y0001479/2), elaidic acid (C18:1(9t); 95%, bought from TRC, lot number: 14-MWC-133-1), stearic acid (C18:0; 100%, bought from EP, lot number: S1340000/4), and arachidic acid (C20:0; 98%, bought from TRC, lot number: 1-KOP-22-1) were used. Acetonitrile (Supelco, lot number I1232830236), methanol (J.T.Baker, lot number 2229705856), and formic acid (Merck, lot number K50073264813) were used as a mobile phase.

2.2. Preparation of Standards and Sample

2.2.1. Preparation of Fatty Acids Stock Solution I

Myristic acid (C14:0), palmitic acid (C16:0), oleic acid (C18:1(9)), elaidic acid (C18:1(9t)), stearic acid (C18:0) and arachidic acid (C20:0) standards were weighed on an analytical balance as 3 mg each and transferred to a 10.0 mL volumetric flask and 6.0 mL of acetonitrile and 2.0 mL of methanol were added and kept in an ultrasonic water bath for 10 minutes. After obtaining a clear solution, the flask was completed to volume with acetonitrile.

2.2.2. Preparation of Fatty Acids Stock Solution II

Approximately 37.5 mg of linoleic acid and oleic acid were weighed, and it was transferred into the 50.0 mL of volumetric flask. Then 20.0 mL of acetonitrile and 2.0 mL of methanol solvents were added, respectively. The volumetric flask was shaken gently to obtain homogenous solution then it was completed to the volume with acetonitrile.

2.2.3. Preparation of Working Standards

To obtain a working solution with a final concentration of 7.5 μg/mL, 2.5 mL of fatty acid stock Solution I and 1 mL of fatty acid stock solution II were transferred into a 100 mL volumetric flask and the volume was completed with acetonitrile.

2.2.4. Preparation of Sample

The outer surface of the product canister was cleaned, dried and its mass was measured on an analytical balance (W1) and kept at -80 ºC for 1 hour. At the end of 1 hour, the canister was cut with scissors and the frozen solution inside was quickly transferred to a volumetric flask. After complete evaporation of HFA (Norfluorane, hfa 134a) gas, the inside of the canister was washed with acetonitrile and the washing solution was added to the same volumetric flask. The volume of the flask was then completed to 10.0 mL with acetonitrile. The cut empty canister was dried thoroughly, and its mass was measured (W2).

2.3. Validation Parameters

2.3.1. Specificity

The performance of a specificity test was undertaken to demonstrate the analytical method's capacity to measure only the intended substances in a given sample. There should not be an extraneous peak at the retention time of active substances peak and known impurities in the chromatograms obtained from diluent and placebo solutions. Impurity peaks should be separated from active substances and each impurity peaks. Working standard- myristic acid, palmitic acid, stearic acid, arachidic acid, oleic acid, linoleic acid, elaidic acid- unspiked, spiked, blank samples were measured by developed method herein. Since the instrument with CA detector could not determine the values of purity angle and purity threshold parameters, the results of these values are not given.

2.3.2. Accuracy and Recovery

In the accuracy parameter of the method, preparations of myristic acid, palmitic acid, stearic acid, arachidic acid, oleic acid, linoleic acid, elaidic acid in the sample solution at LOQ, 100% and 150% levels were studied. Nine samples were prepared, 3 preparations for each level. Each recovery value should be between 90.0% - 110.0%, RSD% ≤ 5.0%. The recovery for the LOQ level should be between 70.0% and 130.0%, RSD% ≤ 15.0%.

2.3.3. Repeatability

The purpose of this test is to demonstrate analysis repeatability. Solutions were prepared according to the mentioned in the leachable analysis method. Six consecutive injections of 1 standard preparation and 1 injection each of six spike sample preparations were made to determine intra-day and interday precision studies. Peak areas obtained from this injections and Relative Standard Deviation (RSD%), Standard deviation (SD), confidence interval (CI 95%) value between them were calculated.

2.3.4. Linearity

Linearity is the study of the test results in the analysis method for determining whether a given concentration range is proportional. In this parameter is evaluated regression equation line in which according to least squares method which is draw area corresponding to concentration and correlation coefficient. At least six solutions were prepared between LOQ, 50%, 80%, 100%, 120% and 150% concentration levels which containing each fatty acids The samples were injected system from lower

concentration to highest concentration. The LOQ level, the highest concentration level, and 100% concentration level were injected 6 times, other concentrations were injected three times into the system.

2.3.5. LOQ and LOD

Signal/Noise (S/N) value is determined by calculating or calculated in the device the value of the Limit of Quantification (LOQ), the Limit of Detection (LOD). For LOQ, the Signal to Noise ratio should be ≥ 10 and for LOD, the Signal to Noise ratio should be ≥ 3.

3. Results and Discussion

3.1. Specificity

The method exhibited the absence of interferences at the retention time of the fatty acid peaks, and all the peaks were pure and had different retention times and relative retention times (Table 2). The chromatograms of the standard mixture and the spike sample are given in Figure 1. The selectivity of the method was evident from the HPLC chromatograms shown in Figures S1 and S11 in supporting information, respectively.

Table 1. Specificity data of developed method

Sample Name	Retention Time	Relative Retention Time	Sample Name	Retention Time	Relative Retention Time
Blank	-	-	**Sample**		
C14:0	7.4	0.37	C14:0	-	-
C18:2(9,12)	10.1	0.51	C18:2(9,12)	-	-
C16:0	12.9	0.65	C16:0	-	-
C18:1(9)	14.3	0.72	C18:1(9)	-	-
C18:1(9t)	15.3	0.77	C18:1(9t)	-	-
C18:0	19.9	1.00	C18:0	-	-
C20:0	26.0	1.31	C20:0	-	-
Mix Standard			**Spike Sample**		
C14:0	7.4	0.37	C14:0	7.4	0.37
C18:2(9,12)	10.1	0.51	C18:2(9,12)	10.1	0.51
C16:0	12.9	0.65	C16:0	12.9	0.65
C18:1(9)	14.3	0.72	C18:1(9)	14.3	0.72
C18:1(9t)	15.2	0.76	C18:1(9t)	15.2	0.76
C18:0	19.9	1.00	C18:0	19.9	1.00
C20:0	27.1	1.36	C20:0	27.1	1.36

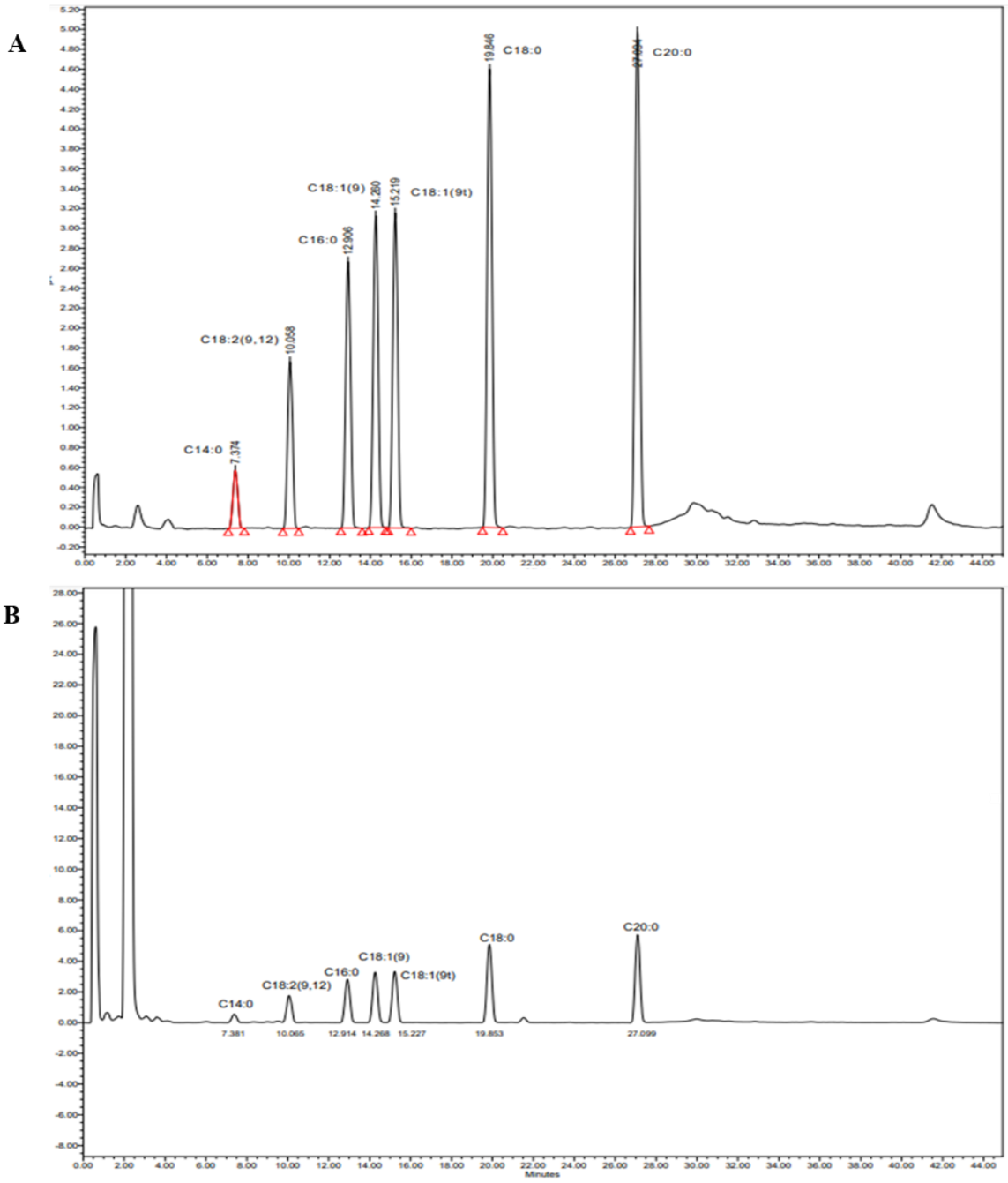

Figure 1. A) The HPLC chromatogram of standard mixture (7.5 μg/mL); B) The HPLC chromatogram of sample.

3.2. Accuracy and Recovery

All impurity recovery results for 100% and 150% are between 90.0% and 110.0%, LOQ results are between 70.0% and 130.0% and meet the acceptance criteria. The result for stearic acid is given in Table 3. Other results are given in the Supporting Information.

Table 2. Accuracy results for stearic acid

Level (%)	Experimental Value (µg/mL)	Theoretical Value (µg/mL)	Recovery (%)	Average (%)	SD	RSD (%)
	0.000820	0.000816	99.5			
LOQ	0.000821	0.000822	100.1	100.1	0.6	0.6
	0.000820	0.000825	100.6			
	0.007450	0.007510	100.8			
100	0.007450	0.007485	100.5	100.7	0.2	0.2
	0.007450	0.007512	100.8			
	0.011175	0.011624	104.0			
150	0.011175	0.011630	104.1	103.8	0.4	0.4
	0.011175	0.011560	103.4			
		Average	101.5			
		SD	1.8			
		% RSD	1.8			
		95% CI	1.9			

3.3. Repeatability

As demonstrated in Table 4, the repeatability parameter relative standard deviation (RSD%) of the sample solution injections was found to be appropriate. A total of six sample results were found to be suitable according to the limit values. The results indicate that the method is reproducible.

Table 3. Repeatability results (µg/canister)

	C14:0	C18:2(9,12)	C16:0	C18:1(9)	C18:1(9t)	C18:0	C20:0
1	71.730	79.023	80.242	79.348	77.486	80.538	79.758
2	67.825	79.422	82.342	79.374	77.400	81.088	78.842
3	69.222	78.010	79.627	78.316	76.590	79.436	76.247
4	68.511	77.830	79.150	78.546	75.791	79.553	77.408
5	68.065	80.649	81.460	80.982	77.957	81.227	79.191
6	68.294	79.971	81.964	80.596	78.249	81.400	79.282
Average	68.941	79.151	80.798	79.527	77.246	80.540	78.455
SD	1.447	1.100	1.310	1.072	0.910	0.861	1.345
% RSD	2.1	1.4	1.6	1.3	1.2	1.1	1.7
95% CI	1.519	1.155	1.375	1.125	0.955	0.904	1.412

3.4. Linearity

Correlation coefficient (R) and determination coefficient (R^2) values were calculated according to the areas given by the solutions prepared at 6 different concentrations (LOQ, 50%, 80%, 100%, 120% and 150%) in the linearity parameter. In Table 5, the LOD and LOQ values of fatty acids for HPLC-CAD are summarized. The method was linear within the range of the LOQ with 150% concentration, and the results are deemed appropriate.

The linearity was determined by analysing the standard solution. Linearity ranges and linear regression equations of the fatty acids are given in Table 2. The correlation coefficients (R^2) were found to be between 0.9920 and 0.9994. The linearity graphics and values of fatty acids can be found in the supporting information.

Table 4. Validation parameters and HLPC-CAD method developed for the leachables fatty acids

	Fatty Acids	Linear regression equation	R^2	LOQ	LOD	Recovery (%)
1	C14:0	y = 1262577435.8433x + 656212.0089	0.9948	10	4	100.4
2	C18:2(9,12)	y = 2560211765.2258x + 1283999.6554	0.9920	12	4	101.7
3	C16:0	y = 6820809942.9622x + 597037.7009	0.9956	10	5	100.1
4	C18:1(9)	y = 7109705075.0544x – 3359514.2098	0.9994	11	5	102.0
5	C18:1(9t)	y = 7850969313.3773x– 2033443.1016	0.9937	11	4	102.7
6	C18:0	y = 10445546725.3598x – 3123109.506(	0.9920	10	5	101.5
7	C20:0	y = 10548647371.5791x – 5114085.940⁴	0.9948	11	4	102.4

In this study, the sample nominal value was determined as 75 µg/canister and the following values were obtained for the fatty acids studied in µg/canister, respectively: myristic acid 68.94, linoleic acid 79.151, palmitic acid 80.798, oleic acid 79.527, elaidic acid 77.246, stearic acid 80.540, and arachidic acid 78.455.

3.5. LOQ and LOD

Table 5 shows the LOQ results. The results are deemed to be suitable, with S/N values ≥ 10. As seen in Table 6, the LOD results are suitable according to the S/N values ≥ 3.

Table 5. LQ data of developed method

Fatty Acids	Concentration (µg/mL)	µg/canister	S/N
C14:0	0.33 µg/mL	3.3 µg/canister	10
C18:2(9,12)	0.345 µg/mL	3.45 µg/canister	12
C16:0	0.12 µg/mL	1.2 µg/canister	10
C18:1(9)	0.75 µg/mL	7.5 µg/canister	11
C18:1(9t)	0.75 µg/mL	7.5 µg/canister	11
C18:0	0.825 µg/mL	8.25 µg/canister	10
C20:0	0.75 µg/mL	7.5 µg/canister	11

Table 6. LOD data of developed method

Fatty Acids	Concentration (µg/mL)	µg/canister	S/N
C14:0	0.1089 µg/mL	1.089 µg/canister	4
C18:2(9,12)	0.1139 µg/mL	1.139 µg/canister	4
C16:0	0.0396 µg/mL	0.396 µg/canister	5
C18:1(9)	0.2475 µg/mL	2.475 µg/canister	5
C18:1(9t)	0.2475 µg/mL	2.475 µg/canister	4
C18:0	0.2723 µg/mL	2.723 µg/canister	5
C20:0	0.2475 µg/mL	2.475 µg/canister	4

4. Conclusions

Leachable analyses act a vital role in ensuring the safety of pharmaceutical products and assessing their compliance with regulatory requirements. Potential impurities, such as fatty acids originating from packaging materials or the manufacturing process, can directly affect the efficacy, stability, and patient safety of pharmaceutical products. Therefore, this study presents an innovative approach to the analysis of leachable impurities in pharmaceutical products by developing a highly sensitive and accurate HPLC/CAD method. Designed for the detection of valve-related leachable fatty acids (myristic acid, linoleic acid, palmitic acid, oleic acid, elaidic acid, stearic acid, and arachidic acid) in metered-dose inhalation products, this method and validation parameter provides a reliable and repeatable analysis process that meets the needs of the industry.

As a result, this study fills an important gap in the quality assurance processes of pharmaceutical products, in terms of patient safety and regulatory requirements. The applications of the developed method might have a significant contribution to the evaluation of the effectiveness of pharmaceutical packaging and storage systems by enhancing the reliability of leachables studies in the pharmaceutical industry.

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References

[1] D. Jenke (2018). Identification, analysis and safety assessment of leachables and extractables, *TrAC Trends Anal. Chem.* **101**, 56-65.
[2] European Medicines Agency (EMA) (2012). Guideline on setting specifications for related impurities in antibiotics.
[3] Product Quality Research Institute (PQRI) (2006). Safety thresholds and best practices for extractables and leachables in orally inhaled and nasal drug products, Product Quality Research Institute: Arlington, VA.
[4] USP-NF ⟨1664⟩ (2015). Assessment of drug product leachables associated with pharmaceutical packaging/delivery systems. https://doi.usp.org/USPNF/USPNF_M7127_02_01.html
[5] D. Skoog, F. Holler and T. Nieman, (1998). Instrumental analysis, Bilim Publishing, Trans: E. Kılıç, F. Köseoğlu, H.Yılmaz, Ankara, Türkiye, pp. 725-768. (In Turkish)
[6] A. A. Wakankar, Y. J. Wang, E. Ma. S. Canova-Davis, D. Schmalzing, J. Grieco, T. Milby, T. Reynolds, K. Mazzarella, E. Hoff, S. Gomez and S. Martin-Moe (2010). On developing a process for conducting extractable-leachable assessment of components used for storage of biopharmaceuticals, *J. Pharm. Sci.* **99**(**5**), 2209-2218.
[7] G. Solomons (2000). Organic chemistry, John Wiley & Sons, Inc, Hoboken, NJ 07030, USA.
[8] M. I. Gurr and A. T. James (1971). An introduction lipid biochemistry, Zahlreiche Abb. Und Tab. London, England.
[9] B. Serpek and L. Kalaycıoğlu (2000). Biochemistry, Nobel Publishing Distribution, Ankara, Türkiye. (In Turkish)
[10] M. Aksoy (2011). Nutrition biochemistry (5th edition), Hatipoglu Publishing House, Türkiye. (In Turkish)
[11] C. Tüzün (1992). Biochemistry, Palme Publications, Ankara, Türkiye. (In Turkish)
[12] I. N. Acworth and W. Kopaciewicz (2017). Charged aerosol detection: A literature review. Charged aerosol detection for liquid chromatography and related reparation techniques, ed: Paul H. Gamache, Thermo Fisher Scientific, Hoboken, NJ 07030, USA, pp. 67–162.

[13] T. Vehovec and A. Obreza (2010). Review of operation of principle and applications of the charged aerosol detector, *J. Chromatogr. A*, **1217**(**10**), 1549-1556.
[14] D. Kiransinh Solanki, S.P. Patel, S. Patel, J. Surati, A. Akabari, D. Sahu and K. Shah (2023). Simultaneous determination of aripiprazole and escitalopram oxalate by HPLC, *J. Chem. Metrol.* **17**(**2**), 128 -138.
[15] T. Patel, S. Padhiyar, V. Bhavsar, J. Upadhyay, V. Prajapati and H. Kela (2023). Development and validation of RP-HPLC method for estimation of Torsemide and Spironolactone in bulk and pharmaceutical dosage forms: a quality by design approach, *J. Chem. Metrol.* **17**(**2**), 148 – 169.
[16] L. Cárdenes, J. H. Ayala, V. González and A. M. Afonso (2002). Fast microwave-assisted dansylation of N-nitrosamines. Analysis by high-performance liquid chromatography with fluorescence detection, *J. Chromatogr. A*, **946** (**1-2**), 133-140.
[17] S. H. Chang, C. C. Chang, L. J. Wang, W. C. Chen, S. Y. Fan, C. Z. Zang and D. Y. Wang (2020). A multi-analyte LC-MS/MS method for screening and quantification of nitrosamines in sartans, *J. Food Drug Anal.* **28**(**2**), 292-301.