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(54) **Title:** ANALYSIS METHOD ENABLING THE DETERMINATION OF THE QUANTITIES OF ALL THE SUBSTANCES IN A SAMPLE UNDER THE SAME CHROMATOGRAPHIC CONDITIONS

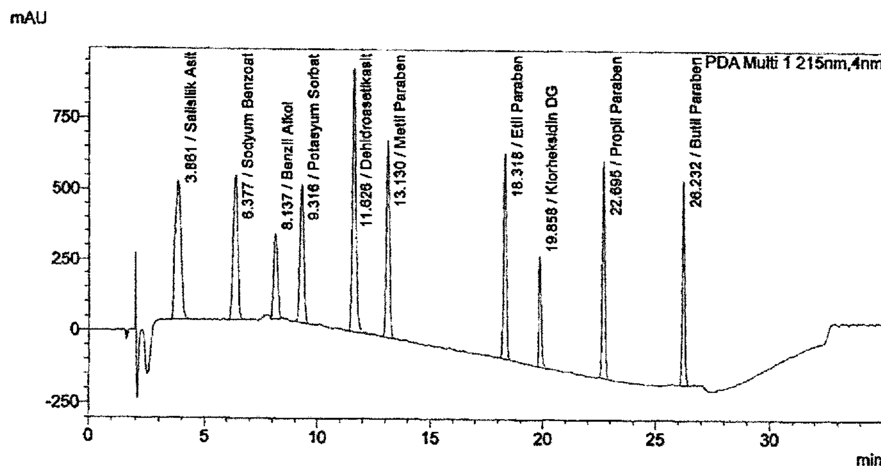

Figure 1

(57) **Abstract:** The subject of the invention relates to an analysis method and an analysis system, which enable the determination of the quantities of the substances in a mixture under the same chromatographic conditions, hence by performing a single feed into a single chromatography column. The analysis method according to the invention basically comprises the steps of obtaining a mobile phase system by mixing 83-87% by volume acetate buffer with a pH of 4.5 and 13- 17% by volume acetonitrile; dissolving the sample to be analyzed in the prepared mobile phase; preparing a column having a length of 250 mm and an inner diameter of 4.6 mm and comprising C18 with a particle size of 5 µm as the stationary phase,injecting into the prepared column a quantity of the sample-mobile phase solution.

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DESCRIPTION

ANALYSIS METHOD ENABLING THE DETERMINATION OF THE QUANTITIES OF ALL THE SUBSTANCES IN A SAMPLE UNDER THE SAME CHROMATOGRAPHIC CONDITIONS

5 **Subject of the Invention**

The subject of the invention relates to an analysis method and an analysis system, which enable the determination of the quantities of the substances in a sample under the same chromatographic conditions, more specifically, via a single method using the high-pressure 10 liquid chromatography, hence by performing a single feed into a single chromatography column.

State of the Art

15 Chromatography is the name given to the processes of separation, which enable the substances in a mixture/sample present in a common suitable solvent medium to be separated by way of dissolution, based on the physical and chemical properties of said substances. There are many chromatographic methods available for use in the state of the art, which are classified according to the mechanisms of separation, the modes of 20 application and the phase types. These methods are based on the principle of the movement of the substances on a stationary phase at various speeds with the help of a mobile phase. The mobile phase is a solvent with various physical and chemical properties, which carries the sample along the stationary phase. The stationary phase, i.e., the column packing material, is the phase with which the substances present in the sample arriving with 25 the mobile phase interact, said substances being retained for different durations at different times, i.e., being retained and slowed down at certain ratios, in the stationary phase. Owing to the retention and separation of the substances, it is possible to separately measure with the help of a detector the quantities of the substances while the substances

are leaving the column. The gas chromatography (GC) and the high-pressure liquid chromatography (HPLC), which are categorized under the column chromatography class and which differ from the other chromatographic methods by their modes of application, are among the main chromatographic methods that are most preferred. In these methods, 5 the stationary phase always consists of “a solid” or of “a liquid layer with which a solid support is impregnated”, whereas the mobile phase consists of “a liquid” or “a gas”.

The gas chromatography (GC) is a method, in which the sample to be analyzed is vaporized and injected to a chromatography column and is carried by a gaseous mobile phase. Unlike the other chromatographic methods, the substances to be analyzed do not interact with 10 the carrier gas phase, with the only function of the gas being to carry the substance/s along the column. The gas chromatography may be applied only to the substances that are volatile or able to be volatilized and that do not decompose at the temperatures used to vaporize them. In case the substances to be subjected to analysis are not volatile, they are rendered volatile by being mixed with various chemicals. On the other hand, in the high-pressure 15 liquid chromatography (HPLC), the mobile phase is a liquid solvent. This is a method, which enables high-efficiency separation of more than one substance in the mixtures and hence allows highly precise and accurate analysis of the quantities of said substances via the same method by performing a single feed of a single sample into a single column, wherein a small injection volume is sufficient for said method to perform these steps, said method having 20 the conditions that do not affect the substances to be analyzed and that are easy to attain and thus having a wide range of application and making it possible to work under the same chromatographic conditions for each substance.

In order to be able to separate with high efficiency the substances in a sample fed into the column and determine with high precision the quantities of said substances by way of 25 application of said chromatographic methods, it is necessary to identify the most suitable method for each substance, including the column dimensions (column length and column inner diameter), type and particle size of the column packing material, mobile phase contents, type of the detector to detect the quantity of the substances as they leave the column and the analysis conditions such as pressure, temperature, flow rate, etc. The ability

to separate each substance with high efficiency and measure the quantity of the same with high precision is directly related to the compatibility between these properties.

Although the methods are available in the state of the art for determining the quantity of each substance in a sample by the use of said chromatographic methods, these methods are of a substance-specific nature. The practices where the quantity of each of a plurality of substances is able to be determined via the same method, hence by using a single column type and performing a single column feed, are not encountered. For example, according to the state of the art, the gas chromatography method is applied for the preservative agent samples, particularly the preservative agent samples comprising salicylic acid, sodium benzoate, potassium sorbate, chlorhexidine digluconate, benzyl alcohol, dehydroacetic acid, butyl paraben, ethyl paraben, methyl paraben and propyl paraben, in order to perform the quantity determination. The method specific to each of these substances is different. Accordingly, the columns with different properties are used for determining the quantity of each substance, wherein the total number of columns used and the number of column feeds performed are the same as the number of the substances whose quantity is to be determined. The procurement of the columns designed for different methods takes months. Due to the conditions of performance of the gas chromatography, the excessive deviations occur between the injections in the analyses, the analysis times for the quantities of the substances are quite long, the overlaps occur in the peaks of some substances and the quantities of such substances are unable to be detected. Because of the drawbacks mentioned above, it is difficult to implement a single method of gas chromatography that enables the analysis of each of the substances present in a sample, especially in the preservative agent samples.

In addition to all these disadvantages, the gas chromatography is performed at high temperatures and some substances lose their stability at very high temperatures. The spectroscopic methods may be additionally required for the determination of some substances. Therefore, this method is restrictive and is not reliable for the separation of the substances in a sample comprising more than one substance with high efficiency and for the quantification of said substances. Furthermore, this method necessitates the use of

additional volatile chemicals and analysis devices according to the characteristics of the substances to be analyzed, resulting in losses of time, labor and cost.

In order to eliminate the above-mentioned drawbacks, an analysis method, via which the separation of more than one substance in a sample is enabled to be performed with high efficiency by the use of a single method, hence by using a single column and by performing a single feed, and via which the quantity of each of the substances is enabled to be determined with high precision, has been developed within the scope of the invention. This analysis has been made possible by the implementation of the high-pressure liquid chromatography (HPLC) method having the chromatographic conditions compatible with one another, such as the column size, mobile phase contents, column packing material type and detector type, which are determined as a result of the experimental studies. Owing to this method, it is made possible to achieve the high-precision measurement, via the same method and via the same feed in the same column, of the quantities of the preservative agents most commonly used in the pharmaceutical and cosmetic industries, such as salicylic acid, sodium benzoate, potassium sorbate, chlorhexidine digluconate, benzyl alcohol, dehydroacetic acid, butyl paraben, ethyl paraben, methyl paraben and propyl paraben, the quantification for which is possible in the state of the art only by using separate methods and by passing the sample through the columns specific to each of said substances. Consequently, the analysis times for these substances are shortened and savings are achieved in time, labor and cost.

Object of the Invention

An object of the invention is to develop an analysis method and an analysis system, which enable the separation of each of the substances in a sample to be performed under the same chromatographic conditions, i.e., by the use of the same method, and enables the determination of the quantity of each substance with high precision.

Another object of the invention is to develop an analysis method and an analysis system, which enable the separation of each of the substances in a preservative agent sample, especially, the substances salicylic acid, sodium benzoate, potassium sorbate, chlorhexidine digluconate, benzyl alcohol, dehydroacetic acid, butyl paraben, ethyl paraben, methyl paraben and propyl paraben most commonly used in the pharmaceutical and cosmetic industries, to be performed under the same chromatographic conditions, i.e., by the use of the same method, and enables the determination of the quantity of each of said substances with high precision.

Another object of the invention is to develop an analysis method and an analysis system, which make it possible to employ the high-pressure liquid chromatography technique and allow the use of a single method, instead of the gas chromatography technique requiring the use of as many methods as the number of substances in a sample, especially for the analysis of the preservative agent samples.

Another object of the invention is to develop an analysis method and an analysis system, which, by eliminating the current requirement to use as many methods as the number of substances, especially for the analysis of the quantities of the substances present in the preservative agent samples, enable the analysis to be completed in a shorter time compared to the state of the art and provides savings in time, labor and cost.

Description of the Figures

Figure 1: A chromatogram obtained by the analysis of an injection of **s**tandard1

Figure 2: A chromatogram obtained by the analysis of an injection of **s**tandard2

Figure 3: A chromatogram obtained by the analysis of a sample

Description of the Tables

Table 1: Gradient system data

Table 2: Quantitative analysis results for the standards

Table 3: Retention times for the substances in the sample

Table 4: Quantitative analysis results for the sample

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Description of the Invention

The subject of the invention relates to an analysis method and an analysis system, which enable the determination of the quantities of the substances in a sample under the same
10 chromatographic conditions, more specifically, via a single method using the high-pressure liquid chromatography, hence by performing a single feed into a single chromatography column.

The sample to be analyzed via the analysis method according to the invention is preferably a sample comprising at least one substance with a concentration of 0.2-0.5 mg/mL. In other
15 words, the analysis method according to the invention is a method suitable for the analysis of only the substances having a concentration value in this concentration range.

The sample to be analyzed via the analysis method according to the invention is a sample preferably comprising at least one preservative agent, preferably the preservative agents salicylic acid, sodium benzoate, potassium sorbate, chlorhexidine digluconate, benzyl
20 alcohol, dehydroacetic acid, butyl paraben, ethyl paraben, methyl paraben and propyl paraben.

The sample to be analyzed via the analysis method according to the invention is a preservative agent sample preferably comprising 0.2 mg/mL Salicylic Acid, 0.2 mg/mL Sodium Benzoate, 0.2 mg/mL Potassium Sorbate, 0.2 mg/mL Benzyl Alcohol, 0.2 mg/mL
25 Dehydroacetic Acid, 0.5 mg/mL Chlorhexidine Digluconate, 0.2 mg/mL Butyl Paraben, 0.2 mg/mL Ethyl Paraben, 0.2 mg/mL Methyl Paraben and 0.2 mg/mL Propyl Paraben.

In order to provide the mentioned ideal chromatographic conditions, i.e., in order to develop the analysis method, the studies to determine said conditions were first conducted, and subsequently, whether these were the conditions suitable for the quantification of each of the substances present in the sample to be analyzed was tested in accordance with the system suitability test.

The high-pressure liquid chromatography (HPLC) is a method, which enables the separation of all the substances in a sample with high efficiency, enables the determination of the quantities of the substances with high precision and accuracy, enables a small injection volume to be sufficient to perform all these, and has conditions that are easily attained and that do not affect the substances to be analyzed. Accordingly, the analysis method is developed in line with the mentioned chromatographic method.

Within the scope of the studies for determining the ideal chromatographic conditions, first the analyses were performed using a column with the length of 150 mm comprising C18 stationary phase, a column with the length of 150 mm comprising C8 stationary phase, a column with the length of 250 mm comprising C8 stationary phase and a column with the length of 150 mm comprising CN type stationary phase and using the mobile phase systems respectively comprising 90% by volume 0.1% phosphoric acid solution and 10% by volume acetonitrile, 85% by volume 0.1% phosphoric acid solution and 15% by volume acetonitrile, 90% by volume phosphate buffer with a pH of 3 and 10% by volume acetonitrile, 80% by volume phosphate buffer with a pH of 3 and 20% by volume acetonitrile, 90% by volume acetate buffer with a pH of 4.5 and 10% by volume acetonitrile, and 85% by volume acetate buffer with a pH of 4.5 and 15% by volume acetonitrile. However, the shapes of the peaks and the peaks observation time points on the chromatograms obtained as a result of the analyses performed using these column-mobile phase combinations and thus the substance separation efficiencies of the columns were not found sufficient.

In order to eliminate the above-mentioned drawbacks, a column, which had a length of 250 mm and an inner diameter of 4.6 mm and which C18 with a particle size of 5 µm as the stationary phase, i.e., the packing material, was developed. As a result of the analyses

performed using this column and the prepared mobile phase systems, it was determined that the peak shapes, the substance separation efficiency of the column and the analysis time were ideal on the chromatograms obtained by the use of this column and the gradient mobile phase system comprising 83-87% by volume, preferably 85% by volume, acetate buffer with a pH of 4.5 and 13-17% by volume, preferably 15% by volume, acetonitrile (initial ratios). Consequently, it was understood that it would be appropriate to perform the analysis by the use of the column, which had a length of 250 mm and an inner diameter of 4.6 mm and which C18 with a particle size of 5 µm as the stationary phase, i.e., the packing material, and of the gradient mobile phase system comprising 83-87% by volume, preferably 85% by volume, acetate buffer with a pH of 4.5 and 13-17% by volume, preferably 15% by volume, acetonitrile (initial ratios). In this way, the ideal chromatographic conditions were achieved, i.e., the ideal method was formed, for enabling the separation of each substance in the sample with high efficiency and the quantification of each substance with high precision and accuracy.

The system suitability test comprises the steps of preparing 2 standard solutions (standard1 and standard2); dissolving each of the standards in the mobile phase system comprising 83-87% by volume acetate buffer with a pH of 4.5 and 13-17% by volume acetonitrile; performing 5 injections of the standard1-mobile phase solution and 2 injections of the standard2-mobile phase solution into the column having a length of 250 mm and an inner diameter of 4.6 mm and comprising C18 with a particle size of 5 µm as the stationary phase, i.e., the packing material; passing each substance in the sample, which is carried by the mobile phase and is separated, through a detector at the outlet of the column; obtaining the quantitative analysis results and the chromatograms; thus determining the retention times of the substances and their concentrations in the standards; and assessing the compatibility of the results with the system suitability requirements.

The system suitability requirements include a resolution value of at most 2% for each substance in the standard1-mobile phase solution and a compatibility of 98-102% between the standard1-mobile phase solution and the standard2-mobile phase solution. The theoretical plate number indicates the efficiency of the column, the resolution value

indicates the efficiency of the chromatography device, and the compatibility between the standards indicates the reliability of the analysis performed.

The standards were prepared by way of obtaining a mobile phase system by preparing the acetate buffer with a pH of 4.5 and mixing 830-870 mL, preferably 850 mL, acetate buffer
5 and 130-170 mL, preferably 150 mL, acetonitrile; introducing 20 mg salicylic acid, 20 mg sodium benzoate, 20 mg potassium sorbate, 20 mg benzyl alcohol, 20 mg dehydroacetic acid, 50 mg chlorhexidine digluconate, 20 mg butyl paraben, 20 mg ethyl paraben, 20 mg methyl paraben and 20 mg propyl paraben to 100 mL solvent; and keeping the ingredients together in a ultrasonic bath until a homogeneous solution is obtained, preferably for 5
10 minutes. The acetate buffer with a pH of 4.5 comprised in the mobile phase system is obtained by dissolving 1.79-1.81 g, preferably 1.8 g, sodium acetate anhydride in 895-905 mL, preferably 900 mL, water; adding to the solution the glacial acetic acid in a quantity sufficient to bring the pH value to 4.5; and complementing the solution to 1000 mL with pure water. The solvent is obtained by mixing identical volumes of acetonitrile and water.
15 In other words, the ratio of the volume of acetonitrile to the volume of water is 1 in the solvent. In a preferred embodiment of the invention, the solvent is obtained by mixing 500 mL acetonitrile and 500 mL water.

1 ml of the standard solution is taken to complement the mobile phase system to 20 ml, and the mobile phase system is stirred and passed through a 0.45 µ RC filter in order to
20 protect the chromatography device by preventing the clogging. The concentrations of salicylic acid, sodium benzoate, potassium sorbate, benzyl alcohol, dehydroacetic acid, chlorhexidine digluconate, butyl paraben, ethyl paraben, methyl paraben and propyl paraben in the final solution (standard solution + mobile phase) are respectively 0.2 mg/mL, 0.2 mg/mL, 0.2 mg/mL, 0.2 mg/mL, 0.2 mg/mL, 0.5 mg/mL, 0.2 mg/mL, 0.2 mg/mL, 0.2
25 mg/mL and 0.2 mg/mL. 20 microliter of the final solution is fed into the column in order to perform the analysis.

The quantitative results and the chromatograms resulting from the analysis of the standards were obtained with the help of the detector. The detector enables the visualization of the

substances exiting the column, the determination of the separation of said substances, and the determination of the quantities of said substances. The substances passing through the detector are recorded by means of at least one recorder and the chromatograms are then generated. The chromatograms obtained from the detectors are today able to be easily interpreted by various computer programs. The system suitability was tested by the assessment of the quantitative results and the chromatograms and the retention times and quantities of the substances were determined. The quantitative results can be seen in the tables below and the chromatograms can be seen in the section of our application providing the figures. The evaluation of the results revealed that the determined chromatographic conditions, i.e., the method, were suitable for the system. It can be seen from the resolution peaks that the substances were separated with high efficiency.

Table 1: Gradient system data

Time (min)	Flow (ml/min)	Mobile Phase A	Mobile Phase B
0	1.5	85	15
5	1.5	85	15
15	1.5	70	30
25	1.5	50	50
30	1.5	85	15
35	1.5	85	15

Table 2: Quantitative analysis results for the standards

Substance Name	Injection Name	Retention Time	Peak Area	Peak Length	Substance Conc.	Resolution (USP)
Salicylic Acid	Standard1_avg	3.861	8727166	491810	0.219	-
	Standard2_avg	3.831	8482475	708430	0.216	-
Sodium Benzoate	Standard1_avg	6.377	7124981	513792	0.204	5.957
	Standard2_avg	6.477	7075585	597101	0.204	8.130

Benzyl Alcohol	Standard1_avg	8.137	3607655	300155	0.273	5.061
	Standard2_avg	8.250	3492041	309107	0.270	5.634
Potassium Sorbate	Standard1_avg	9.316	5635805	492661	0.203	3.694
	Standard2_avg	9.415	5552914	531050	0.202	3.918
Dehydroacetic Acid	Standard1_avg	11.626	9623628	928318	0.234	7.765
	Standard2_avg	11.761	9499406	937278	0.232	8.313
Methyl Paraben	Standard1_avg	13.130	6523324	698744	0.208	5.563
	Standard2_avg	13.204	6419146	719044	0.207	5.488
Ethyl Paraben	Standard1_avg	18.318	5952490	726325	0.202	21.086
	Standard2_avg	18.349	5912295	726406	0.201	21.377
Chlorhexidine DG	Standard1_avg	19.858	2809770	388754	0.116	7.034
	Standard2_avg	19.664	2797152	405051	0.116	6.165
Propyl Paraben	Standard1_avg	22.695	5733489	768779	0.208	13.567
	Standard2_avg	22.697	5676839	763367	0.207	14.832
Butyl Paraben	Standard1_avg	26.232	5229632	721208	0.203	16.845
	Standard2_avg	26.218	5185834	715290	0.202	16.697

Table 3: Retention times for the substances in the sample

Substance Name	**Retention Time (min)**
Salicylic Acid	3.8
Sodium Benzoate	6.4
Benzyl Alcohol	8.1
Potassium Sorbate	9.3
Dehydroacetic Acid	11.6
Methyl Paraben	13.1
Ethyl Paraben	18.3
Chlorhexidine Digluconate	20.0
Propyl Paraben	22.7
Butyl Paraben	26.2

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Consequently, the analysis method according to the invention basically comprises the steps of

- **i.** obtaining a mobile phase system by mixing 83-87% by volume acetate buffer with a pH of 4.5 and 13-17% by volume acetonitrile,
- **ii.** dissolving the sample to be analyzed in the prepared mobile phase,
- **iii.** preparing a column having a length of 250 mm and an inner diameter of 4.6 mm and comprising C18 with a particle size of 5 µm as the stationary phase, i.e., the packing material,
- **iv.** injecting into the prepared column a quantity of the sample-mobile phase solution that would not cause clogging, and
- **v.** passing each substance in the sample, which is carried by the mobile phase and is separated, through the detector at the outlet of the column.

In a preferred embodiment of the analysis method according to the invention, the mobile phase system is obtained by mixing 85% by volume acetate buffer with a pH of 4.5 and 15% by volume acetonitrile.

In a preferred embodiment of the analysis method according to the invention, the sample-mobile phase solution is injected into the prepared column at a pressure in the range of 120-130 bars, preferably at a pressure of 125 bars.

In a preferred embodiment of the analysis method according to the invention, the sample-mobile phase solution is injected into the prepared column in 35 minutes.

In a preferred embodiment of the analysis method according to the invention, the volume of the sample-mobile phase solution injected into the prepared column is 20 µL.

In a preferred embodiment of the analysis method according to the invention, the ratio of the volume of the sample to the volume of the mobile phase is 1:19 in the sample-mobile phase solution. In a preferred embodiment of the analysis method according to the invention, 20 mL sample-mobile phase solution comprises 1 mL sample.

In a preferred embodiment of the analysis method according to the invention, the flow rate of the sample-mobile phase solution inside the column is 1.4-1.6 mL/min, preferably 1.5 mL/min.

In a preferred embodiment of the analysis method according to the invention, the
5 temperature of the prepared column is 38-42°C, preferably 40°C.

In a preferred embodiment of the analysis method according to the invention, the tray temperature of the prepared column is 13-17°C, preferably 15°C.

In a preferred embodiment of the analysis method according to the invention, each substance in the sample, which is carried by the mobile phase and is separated, is passed
10 through a PDA detector emitting light in the UV and visible region or an ultraviolet/visible region detector.

In a preferred embodiment of the analysis method according to the invention, the analysis is performed at the wavelength of 210-220 nm, preferably 215 nm.

15 **Table 4:** Quantitative analysis results for the sample

Substance Name	Retention Time	Peak Area	Peak Height	Substance Concentration	Resolution (USP)
Salicylic Acid	3.688	8467002	714066	0.215	-
Sodium Benzoate	6.446	6992262	588413	0.201	8.475
Benzyl Alcohol	8.274	3514130	307737	0.272	5.791
Potassium Sorbate	9.416	5478207	526181	0.199	3.838
Dehydroacetic Acid	11.805	94445175	934992	0.231	8.466
Methyl Paraben	13.226	6423198	717484	0.207	5.397
Ethyl Paraben	18.368	5902174	725041	0.201	21.355
Chlorhexidine DG	19.756	2774040	406729	0.115	6.499
Propyl Paraben	22.713	5728873	763701	0.208	14.424
Butyl Paraben	26.228	5257779	724051	0.204	16.652

The calculations for the determination of the quantities of the substances in the sample
20 were performed using the formula below. Examination of the quantitative results obtained from the analysis of the sample, comparison of these results to the quantitative results for

the standards and the examination of the peaks revealed that the analysis was performed with high precision and accuracy and the suitability with the system was achieved.

$$\% \text{ Result} = \frac{\text{An}}{\text{As}} \text{x} \frac{\text{Ms}}{100} \text{x} \frac{1}{20} \text{x} \frac{100}{\text{Mn}} \text{x} \frac{20}{1} \text{x} \frac{\text{P}}{100} \text{x} 100$$

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An: Area of the peaks of salicylic acid, sodium benzoate, potassium sorbate, benzyl alcohol, dehydroacetic acid, chlorhexidine digluconate, butyl paraben, ethyl paraben, methyl paraben and propyl paraben in the test solution

As: Area of the peaks of salicylic acid, sodium benzoate, potassium sorbate, benzyl alcohol,
10 dehydroacetic acid, chlorhexidine digluconate, butyl paraben, ethyl paraben, methyl paraben and propyl paraben in the standard solution

Ms: Standard weight (mg)

Mn: Sample weight (mg)

P: Standard Potency (%)

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A preferred embodiment of the analysis method according to the invention comprises the process steps of

i. obtaining a mobile phase system by mixing 85% by volume acetate buffer with a pH of 4.5 and 15% by volume acetonitrile,

20 **ii.** dissolving a sample, which comprises salicylic acid, sodium benzoate, potassium sorbate, benzyl alcohol, dehydroacetic acid, chlorhexidine digluconate, butyl paraben, ethyl paraben, methyl paraben and propyl paraben each at a concentration in the range of 0.2-0.5 mg/mL, in the prepared mobile phase,

iii. preparing a column having a length of 250 mm and an inner diameter of 4.6 mm and comprising C18 with a particle size of 5 µm as the stationary phase, i.e., the packing material,

iv. injecting 20 microliter of the sample-mobile phase solution into the column, which has a temperature of 40°C and a tray temperature of 15°C, at a pressure of 125 bars in 35 minutes, and

v. passing each substance in the sample, which is carried by the mobile phase and is separated, through the detector at the outlet of the column.

The invention further relates to an analysis system, which enables the substances in a sample to be analyzed by the use of high-pressure liquid chromatography and under the same chromatographic conditions, said analysis system comprising a mobile phase that comprises 83-87% by volume acetate buffer with a pH of 4.5 and 13-17% by volume acetonitrile and a column having a length of 250 mm and an inner diameter of 4.6 mm and comprising C18 with a particle size of 5 µm as the stationary phase, i.e., the packing material.

It is understood as a result of the evaluations made that the method disclosed in the invention is an analysis method, which enables the high-efficiency separation of more than one substance present in a sample by the use of a single method, hence by employing a single column and by performing a single feed, and which makes it possible to determine the quantity of each of said substances with high precision. This analysis has been made possible by the implementation of the high-pressure liquid chromatography (HPLC) method under the chromatographic conditions compatible with one another, such as the dimensions of the column, the properties of the mobile phase, the properties of the stationary phase, i.e., the column packing material, and the type of the detector. Owing to this method, it is made possible to achieve the high-precision measurement, under the same conditions, i.e., via the same method and in the same column, of the quantities of the

preservative agents such as salicylic acid, sodium benzoate, potassium sorbate, chlorhexidine digluconate, benzyl alcohol, dehydroacetic acid, butyl paraben, ethyl paraben, methyl paraben and propyl paraben, the quantification for which is currently possible only by using separate methods of gas chromatography and by passing the sample
5 through the columns specific to each of said substances. Consequently, the analysis times for these substances are shortened and savings are achieved in time, labor and cost.

CLAIMS

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1. An analysis method, which enables the substances in a sample to be analyzed by the use of the high-pressure liquid chromatography and under the same chromatographic conditions, characterized in that the analysis method comprises the steps of

- **i.** obtaining a mobile phase system by mixing 83-87% by volume acetate buffer with a pH of 4.5 and 13-17% by volume acetonitrile,
- **ii.** dissolving the sample to be analyzed in the prepared mobile phase,
- **iii.** preparing a column having a length of 250 mm and an inner diameter of 4.6 mm and comprising C18 with a particle size of 5 µm as the stationary phase, i.e., the packing material,
- **iv.** injecting the sample-mobile phase mixture into the prepared column and
- **v.** passing each substance in the sample, which is carried by the mobile phase and is separated, through the detector at the outlet of the column.

2. An analysis method according to Claim 1 characterized in that the mobile phase system is obtained by mixing 85% by volume acetate buffer with a pH of 4.5 and 15% by volume acetonitrile.

3. An analysis method according to any one of the preceding claims characterized in that the acetate buffer is obtained by a method comprising the steps of

- **i.** dissolving at least one acetate compound in a quantity of 1.79-1.81 g in 895-905 mL water,
- **ii.** adding to the obtained solution the glacial acetic acid in a quantity sufficient to bring the pH value to 4.5 and
- **iii.** complementing the solution to 1000 mL with pure water.

4. An analysis method according to Claim 3 characterized in that 1.8 mg of at least one acetate compound is dissolved in 900 mL water.

5. An analysis method according to Claim 3 or Claim 4 characterized in that the acetate compound is sodium acetate anhydride.

6. An analysis method according to any one of the preceding claims characterized in that the sample-mobile phase mixture is injected into the prepared column at a pressure in the range of 120-130 bars.

7. An analysis method according to any one of Claims 1-5 characterized in that the sample-mobile phase solution is injected into the prepared column at a pressure of 125 bars.

8. An analysis method according to any one of the preceding claims characterized in that the sample-mobile phase solution is injected into the prepared column in 35 minutes.

9. An analysis method according to any one of the preceding claims characterized in that 20 µL of the sample-mobile phase solution is injected into the prepared column.

10. An analysis method according to any one of the preceding claims characterized in that the flow rate of the sample-mobile phase solution inside the column, into which it is injected, is 1.4-1.6 mL/min.

11. An analysis method according to any one of Claims 1-9 characterized in that the flow rate of the sample-mobile phase solution inside the column, into which it is injected,is 1.5 mL/min.

12. An analysis method according to any one of the preceding claims characterized in that the temperature of the prepared column is 38-42°C.

13. An analysis method according to any one of Claims 1-11 characterized in that the temperature of the prepared column is 40°C.

14. An analysis method according to any one of the preceding claims characterized in that the tray temperature of the prepared column is 13-17°C.

15. An analysis method according to any one of Claims 1-13 characterized in that the tray temperature of the prepared column is 15°C.

16. An analysis method according to Claim 1 characterized in that 20 µL sample-mobile phase solution is injected into the column, which has a temperature of 38-42°C and a tray temperature of 13-17°C, at a pressure in the range of 120-130 bars in 35 minutes such that the flow rate will be 1.4-1.6 mL/min.

17. An analysis method according to Claim 1 characterized in that 20 µL sample-mobile phase solution is injected into the column, which has a temperature of 40°C and a tray temperature of 15°C, at a pressure of 125 bars in 35 minutes such that the flow rate will be 1.5 mL/min.

18. An analysis method according to any one of the preceding claims characterized in that the detector, through which each substance in the sample passes, is a PDA detector emitting light in the UV and visible region.

19. An analysis method according to any one of Claims 1-17 characterized in that the detector, through which each substance in the sample passes, is an ultraviolet/visible region detector emitting light in the UV and visible region.

20. An analysis method according to any one of the preceding claims characterized in that the analysis is performed at the wavelength of 210-220 nm.

5 **21.** An analysis method according to any one of Claims 1-19 characterized in that the analysis is performed at the wavelength of 215 nm.

22. An analysis method according to any one of the preceding claims characterized in that the ratio of the volume of the sample to the volume of the mobile phase is 1:19
10 in the sample-mobile phase solution.

23. An analysis method according to any one of the preceding claims characterized in that the analysis method is a method, which enables the substances present at a concentration of 0.2-0.5 mg/mL in a sample to be analyzed.

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24. An analysis method according to any one of the preceding claims characterized in that the substances in the sample are the preservative agents.

25. An analysis method according to any one of the preceding claims characterized in
20 that the substances in the sample are salicylic acid, sodium benzoate, potassium sorbate, benzyl alcohol, dehydroacetic acid, chlorhexidine digluconate, butyl paraben, ethyl paraben, methyl paraben and propyl paraben.

26. An analysis method according to any one of Claims 1-24 characterized in that the
25 substances in the sample are 0.2 mg/mL salicylic acid, 0.2 mg/mL sodium benzoate, 0.2 mg/mL potassium sorbate, 0.2 mg/mL benzyl alcohol, 0.2 mg/mL dehydroacetic acid, 0.5 mg/mL chlorhexidine digluconate, 0.2 mg/mL butyl paraben, 0.2 mg/mL ethyl paraben, 0.2 mg/mL methyl paraben and 0.2 mg/mL propyl paraben.

27. An analysis system, which enables the substances in a sample to be analyzed by the use of high-pressure liquid chromatography and under the same chromatographic conditions, characterized in that the analysis system comprises a mobile phase that comprises 83-87% by volume acetate buffer with a pH of 4.5 and 13-17% by volume
5 acetonitrile and a column having a length of 250 mm and an inner diameter of 4.6 mm and comprising C18 with a particle size of 5 µm as the stationary phase.

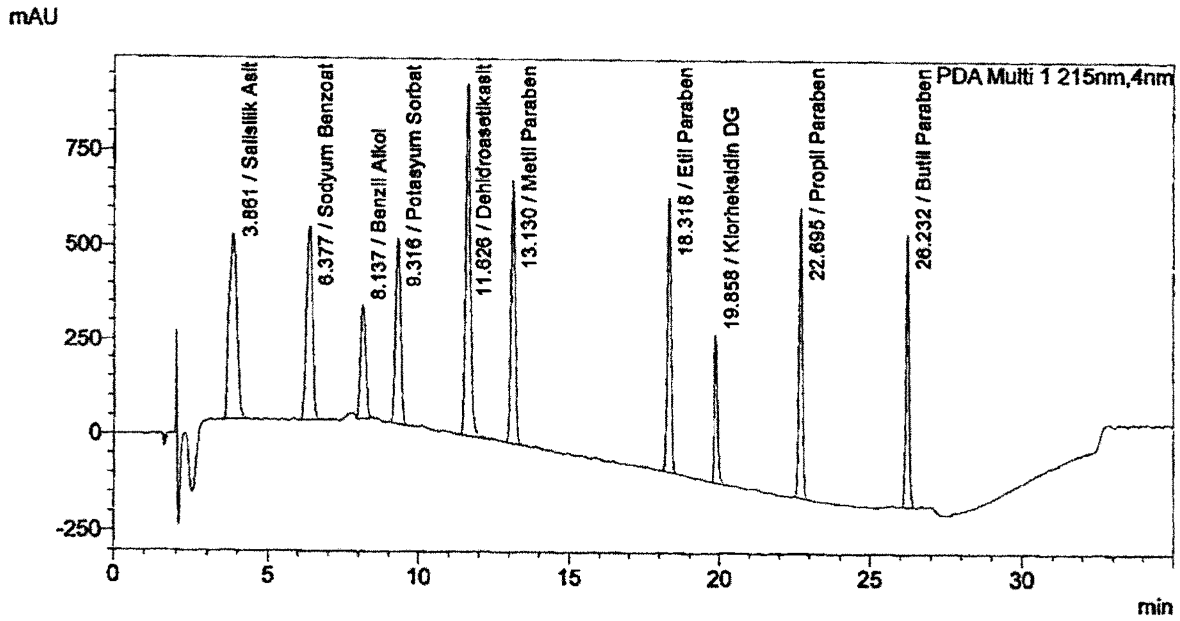

Figure 1

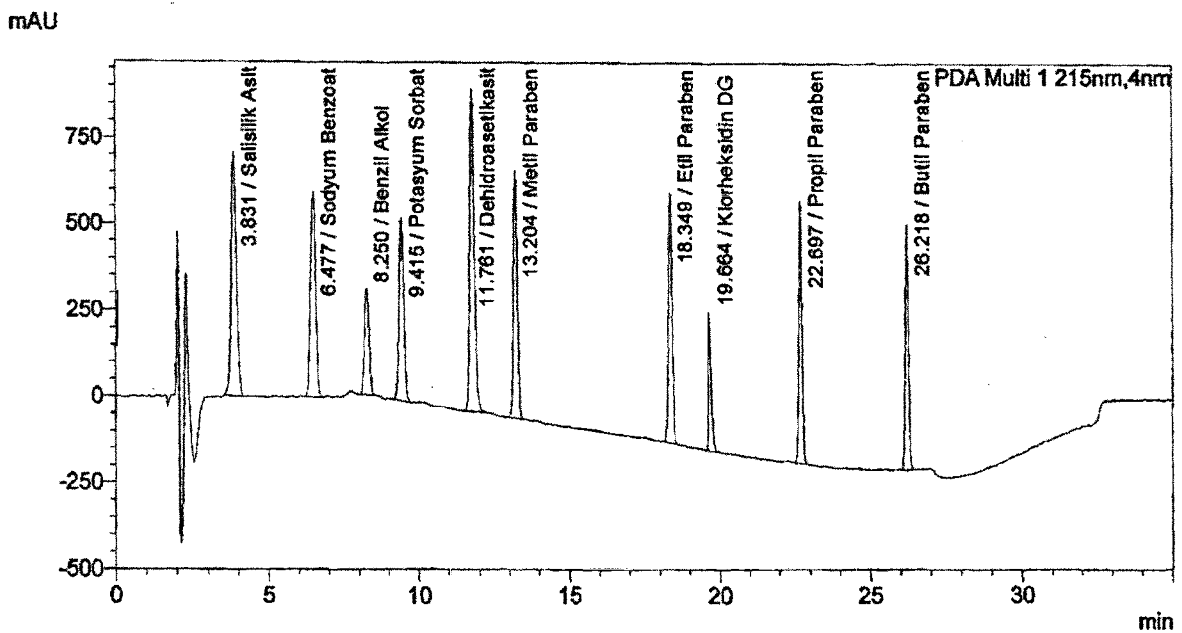

Figure 2

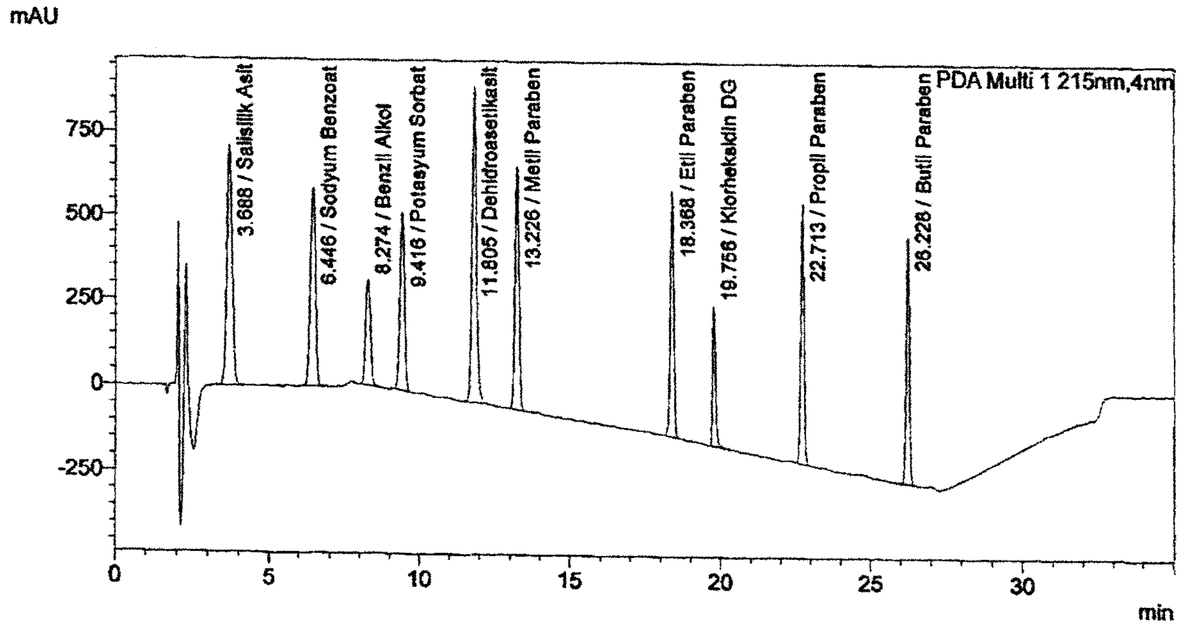

Figure 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/TR2023/051286

A. CLASSIFICATION OF SUBJECT MATTER

G01N 30/02 (2006.01)i; G01N 30/74 (2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-INTERNAL, PUBMED & KEYWORDS: HPLC, chromatography, preservatives, simultaneous, acetonitrile, acetate, C18

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Khosrokhavar R. et al. Simultaneous determination of preservatives (sodium benzoate and potassium sorbate) in soft drinks and herbal extracts using high-performance liquid chromatography (HPLC). Journal of Medicinal Plants 9.35 (2010): 80-87 2010 Abstract, page 81	1-27
X	CN 106645603 A (UNIV GUANGXI NATIONALITIES) 10 May 2017 (2017-05-10) Abstract, paragraphs 25,27,73,81	1-27
X	Marengo E, Gennaro MC, Gianotti V. A simplex-optimized chromatographic separation of fourteen cosmetic preservatives: analysis of commercial products. J Chromatogr Sci. 2001 Aug;39(8):339-44. doi: 10.1093/chromsci/39.8.339 August 2001 Abstract, pages 339-342	1-27
X	Aoyama A, Doi T, Tagami T, Kajimura K. Simultaneous determination of 11 preservatives in cosmetics by high-performance liquid chromatography. J Chromatogr Sci. 2014 Oct;52(9):1010-5. doi: 10.1093/chromsci/bmt144 06 October 2013 (2013-10-06) Abstract, pages 1010-1012	1-27

☑ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

* Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "D" document cited by the applicant in the international application
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- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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Date of the actual completion of the international search	Date of mailing of the international search report
22 March 2024	**22 March 2024**

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/TR2023/051286

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Pylypiw HM Jr, Grether MT. Rapid high-performance liquid chromatography method for the analysis of sodium benzoate and potassium sorbate in foods. J Chromatogr A. 2000 Jun 23;883(1-2):299-304. doi: 10.1016/s0021-9673(00)00404-0 26 June 2000 (2000-06-26) Abstract, pages 300 and 301	1-27

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/TR2023/051286

Patent document cited in search report	Publication date (day/month/year)	Patent family member(s)	Publication date (day/month/year)
CN 106645603 A	10 May 2017	CN 114137140 A	04 March 2022