# CHARLES UNIVERSITY IN PRAGUE

## FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

Department of Pharmaceutical Chemistry and Drug Analysis


## Evaluation of selected pharmaceuticals by HPLC with spectrophotometric detection

MASTER'S THESIS

## Hodnocení vybraných léčiv pomocí HPLC se spektrofotometrickou detekcí

DIPLOMOVÁ PRÁCE


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Vedoucí práce



Hradec Králové 2013

I hereby declare that this thesis is my genuine work, created under the guidance of my supervisor RNDr. Milan Mokrý, CSc. and consultant Ing. Lubomír Galla. All information sources and publications used are properly cited.

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Kateřina Dittrichová

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*Dedicated to*

*Ing. Zbyněk Pospíšil*

# Abstract

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Student: Kateřina Dittrichová
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Title of master's thesis:

**Evaluation of selected pharmaceuticals by HPLC with spectrophotometric detection**

A liquid-chromatographic method was developed for the simultaneous determination of dexpanthenol, phenoxyethanol, and preservatives (methyl-, ethyl-, propyl-, isobutyl-, and butylparaben). Separation of these compounds was performed on column Discovery $C_{18}$ (5 μm, 150 mm × 4.6 mm I.D.) by isocratic elution with a potassium dihydrogen phosphate buffer (0.01 mol $l^{-1}$, pH adjusted to 2.5 with a phosphoric acid 85 %) and an acetonitrile (67:33, v/v) at the flow rate of 1.00 ml $min^{-1}$. The injection volume of 5 μl was used. The diode array detector operated at two wavelengths (210 nm for dexpanthenol and 254 nm for phenoxyethanol and preservatives). Three basic system suitability parameters were evaluated: the number of the theoretical plates of all compounds was greater than 6000 units, determined symmetry factors of all peaks were in interval from 1.1 to 1.3, and calculated values of resolution were greater than 1.5. Accuracy, precision, linearity, specificity, and selectivity tests were satisfactorily performed. The method showed good recovery from 98.00 % to 101.00 % for all compounds. From the linearity experiment, the correlation coefficient was at least 0.99900 for all compounds. The method was successfully applied to the determination of dexpanthenol, phenoxyethanol, and preservatives in gel based on acrylamide.

# Souhrn

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**Hodnocení vybraných léčiv pomocí HPLC se spektrofotometrickou detekcí**

Pro souběžné stanovení dexpanthenolu, fenoxyethanolu a konzervačních látek (methyl-, ethyl-, propyl-, isobutyl- a butylparabenu) byla vyvinuta HPLC metoda. Tyto látky byly separovány na koloně Discovery $C_{18}$ (5 µm, 150 mm × 4,6 mm I.D.) isokratickou elucí mobilní fází, která obsahovala hydrogenfosforečnan draselný (0,01 $mol.l^{-1}$, pH upraveno kyselinou fosforečnou 85 % na hodnotu 2,5) a acetonitril (67:33, v/v), a rychlostí 1,00 $ml.min^{-1}$. Objem nástřiku byl 5 µl. Diode array detektor pracoval při dvou vlnových délkách (210 nm pro dexpanthenol a 254 nm pro fenoxyethanol a konzervační látky). Byly hodnoceny tři základní parametry systému vhodnosti: počet teoretických pater byl pro všechny látky vyšší než 6000 jednotek, stanovené faktory symetrie všech látek spadaly do intervalu 1,1 až 1,3 a vypočtené hodnoty rozlišení byly vyšší než 1,5. Testy správnosti, přesnosti, linearity, specifity a selektivity metody byly vhodně provedeny. Výtěžnost metody se pohybovala v intervalu od 98.00 % do 101.00 % pro všechny látky. Z testu linearity bylo patrné, že korelační koeficient všech látek byl minimálně 0.99900. Metoda byla úspěšně aplikována na stanovení dexpanthenolu, fenoxyethanolu a konzervačních látek v akrylamidovém gelu.

# Contents

# Chapter 1 Introduction

High-Performance Liquid Chromatography (HPLC) is the most progressive analytical method available, with a wide application in analyses of pharmaceuticals, cosmetics, foods, and beverages. HPLC is, relative to other analytical procedures, a very effective, selective, and reliable method for identification of active pharmaceutical ingredients (API) or preservatives, assay, and testing for impurities. Nowadays, there is a growing need for precise determination methods, thus requirements and guidelines for validating these methods have been developed. The International Conference on Harmonisation (ICH) published *Validation of Analytical Procedures: Text and Methodology Q2(R1)*[1] which is a set of recommendations for pharmaceutical industrial companies. According to this guideline the presented method for the determination of dexpanthenol, phenoxyethanol, and preservatives was validated.

Dexpanthenol is a precursor of pantothenic acid (vitamin $B_5$), which is necessary for the synthesis of coenzyme A, an essential coenzyme. Dexpanthenol is commonly used as a supportive treatment of mucus affects (bronchitis, tracheitis, laryngitis, pharyngitis, stomatitis, etc.) and it also externally supports epithelization of ulcers, burn, and scratch wounds. Dexpanthenol is usually formulated in multi-vitamin preparations, parenterals, and some local cosmetic preparations.[2]

Parabens (4-hydroxybenzoic acid esters) are widely used as antimicrobial preservatives in pharmaceutical products and local cosmetics. Recently, preservatives, especially parabens, received intense attention because of their possible adverse effects in humans. As a result, a fast, simple, and accurate method of analysis is necessary.[3]

# Chapter 2 Objective of the thesis

The aim of this thesis is to develop, validate, and apply an optimal liquid-chromatographic method for qualitative and quantitative simultaneous determination of dexpanthenol, phenoxyethanol, and preservatives, such as methylparaben, ethylparaben, propylparaben, butylparaben, and isobutylparaben in farmaceutical preparation.

# Chapter 3 Theoretical part

## 3.1. Chromatographic process

In 1903, Mikhail Semyonovich Tswett was the first scientist who described chromatography as the physicochemical method of separation of complex mixtures.[4] Later, in 1910 in his Doctor of Science dissertation, he explained chromatography as a fractional adsorption process that is based on different affinities between molecules of the different analytes and surface of the porous material. Therefore, analytes with stronger affinities are more retained and the equilibrium between the phases and the sample mixture is reached gradually in multiple repetitions of the chromatographic process.[5]

The International Union of Pure and Applied Chemistry in *Nomenclature for Chromatography* (IUPAC Recommendations, 1993) define chromatography as:

> '**Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (the stationary phase) while the other (the mobile phase) moves in a definite direction.'**[6]

Chromatography is a separation process based on forced transport of the mobile phase carrying the sample mixture through the porous material (column or plane). The differences in the interactions between the mobile phase, the sample mixture and the stationary phase are the main principles of the chromatography.

The HPLC theory can be subdivided into two different aspects: thermodynamic and kinetic. The thermodynamic aspect explains the responsibility of the sample retention in the column as determinative of the peak position on the chromatogram. Kinetic aspect of the chromatographic zone is responsible for the band broadening which explains the width of chromatographic peak.[7]

The HPLC is always used for identification of an active pharmaceutical ingredient (API) or preservatives, assay, and testing for impurities. Main peaks

of chromatogram are very often the API or preservatives, possible smaller peaks can be undesirable impurities (residues of excipients or disintegrated product).[8]

## 3.2. Classification

All chromatographic methods are not suitable for all purposes; each method has its advantages and disadvantages. Planar methods are suitable for identification and screening purposes because of their simplicity, effectiveness, and low price, but are not commonly used for quantitative determinations. Classical column methods are valuable for quantitative evaluation. Also, these methods do not require a special apparatus but this mode of separation is often very time-consuming. High-pressure liquid and gas chromatographic methods require an expensive specialized apparatus. These separations are rapid, very effective, and are suitable for precise qualitative and quantitative determination of components of a sample mixture. The lack of universally applicable methods of detection is very limiting.[9]

### 3.2.1. Nature of Mobile Phase

#### *3.2.1.1. Gas Chromatography*

Gas chromatography (GC), similar to liquid chromatography, is a very sensitive analytical method with wide applications in the pharmaceutical industry. The mobile phase is an inert gas that carries the vaporized sample. GC permits very efficient separation and determination of very low amounts of samples. However, GC has its limitations. It is used primarily for analyzing gases and sufficiently volatile compounds that do not decompose at higher temperatures. Only 20% of these organic compounds are known to be satisfactorily separated by GC, without a prior chemical modification of the sample, called derivatization.[8,10,11]

#### *3.2.1.2. Liquid Chromatography*

Liquid chromatography (LC) can be subdivided into two types: *liquid-solid chromatography* (LSC), if the stationary phase is a solid material, and

*liquid-liquid chromatography* (LLC), if the stationary phase is a liquid. The mobile phase is a stable liquid that is transported through the stationary phase. LC is not limited by sample volatility or thermal stability.

Very difficult separations are more often performed by LC than by GC, in that it uses two chromatographic phases in LC for selective interaction with sample molecules versus only one in GC. A greater variety of unique column packings (stationary phases) and lower separation temperatures can be used in LC.[11,12]

### 3.2.2. Composition of Mobile Phase

#### *3.2.2.1. Isocratic elution*

The whole chromatographic separation is carried out using a mobile phase with a constant composition, i.e. with a constant eluting strength.[10]

#### *3.2.2.2. Stepwise elution*

After separation and complete elution of some components with stated mobile phase with lower eluting strength, the composition and eluting strength are changed stepwise and the rest of the components are thus eluted.[10,13]

#### *3.2.2.3. Gradient elution*

The eluting strength is gradually increased during the separation according to a program. The gradient of the mobile phase composition can be linear or non-linear. This type of elution can be used for more complicated mixtures for faster analysis.[10,13]

### 3.2.3. Arrangement of Stationary Phase

#### *3.2.3.1. Planar chromatography*

In paper chromatography (PC), the stationary phase is a sheet of paper consisting of suitable parameters, which may sometimes be impregnated with a liquid phase that is immiscible with the mobile phase. Nowadays, the PC is obsolete.[9]

In thin-layer chromatography (TLC), the stationary phase is a thin, homogenous layer (usually about 0.24 mm thick) of a dry, finely powdered material applied to a suitable support made of glass, aluminium, or plastic. Movement across the surface of the plate covered with the stationary phase is caused by capillary forces. The TLC is widely used for identification in Pharmacopoeial monographs.[8,9]

#### *3.2.3.2. Column chromatography*

In column chromatography, the stationary phase is filled in a column and it uses gravity or a high pressure as the force of the separation process. Many types of columns with different stationary phases are available.

Column chromatography can be subdivided into the classical column chromatography, the extraction chromatography (SPE), and the high-performance liquid chromatography (HPLC). The HPLC is the more widely used column separation method for analytical purposes.

The HPLC technique, as compared with the classical LC technique, is characterized by:

- small diameter (4.6 mm), stainless steel, glass or titanium columns,
- column packing with very small (3.5 and 10 µm) particles,
- relatively high inlet pressures and controlled flow of the mobile phase,
- continuous flow detectors capable of handling small flow rates and detecting very small amounts,
- high resolution,
- rapid analysis.[8,14,15]

### 3.2.4. Mechanism of Separation

#### *3.2.4.1. Adsorption Chromatography*

The principles of adsorption chromatography (normal-phase chromatography, NPC) are known from the principles of classical column chromatography and thin-layer chromatography. Adsorption chromatography explores the differences in the strength of the polar interactions of the compounds in the sample mixture with a stationary phase. The more polar a molecule, the more strongly it will be adsorbed by a polar stationary phase.

Similarly, the more non-polar a molecule, the more strongly it will be adsorbed by a non-polar stationary phase.

The two most common adsorbents used in chromatography are porous alumina and porous silica gel. The surface of these stationary phases is covered with a dense population of OH groups which makes these surfaces highly polar. Other less often used stationary phases are carbon, magnesium oxide, and various carbonates.[7,16,17]

#### *3.2.4.2. Ion-exchange chromatography*

Ion-exchange chromatography (IEC), as indicated by its name, is based on the different affinities of the sample ions for the oppositely charged ionic centers in the resin or adsorbed counter ions in the hydrophobic stationary phase. The method is suitable for separating, e.g. amino acids, ionic metabolic products or organic ions.[7,16]

#### *3.2.4.3. Ion-pair chromatography*

Ion-pair chromatography may also be used for the separation of ionic compounds and overcomes certain problems inherent in the ion-exchange method. Ionic sample molecules are 'masked' by a suitable counter ion. The main advantages are, firstly, that the widely available reversed-phase system can be used, so no ion exchanger is needed, and, secondly, acids, bases and neutral products can be analyzed simultaneously.[16]

#### *3.2.4.4. Partition chromatography*

When partition chromatography is based on the different solubilities between two immiscible liquids, it is known as liquid-liquid chromatography (LLC). Between a liquid and a gas, it is known as gas-liquid chromatography (GLC). The main condition is absolute immiscibility of mobile and stationary phases. Partition chromatography is impossible to perform in practical use because a part of the stationary phase can be washed out. Therefore, the stationary phases bonded to solid inert support are used instead.[8,18]

#### *3.2.4.5. Size-exclusion chromatography*

Size-exclusion chromatography (SEC) can be subdivided into gel permeation chromatography (with organic solvents) and gel filtration chromatography (with aqueous solutions). The principle of the SEC is separation of molecules by size, i.e. according to molecular mass. The largest molecules are eluted first and the smallest molecules last. This is the best method to choose when a mixture contains compounds with a molecular mass difference of at least 10 %.[16]

#### *3.2.4.6. Affinity chromatography*

In this case, highly specific biochemical interactions provide the means of separation. The stationary phase contains specific groups of molecules that can only adsorb the sample if certain steric and charge-related conditions are met. Affinity chromatography can be used to isolate proteins (enzymes as well as structural proteins), lipids, etc., from complex mixtures without involving any great expenditure.[16]

### 3.2.5. Mode of Separation

#### *3.2.5.1. Frontal Chromatography*

In frontal chromatography the sample is introduced continuously into the column. Only the least retained component exits the column in pure form and can, therefore, be isolated; all other sample components exit the column as mixed zones. The frontal chromatography can also be called adsorptive filtration in that it can be used for the purpose of filtration.[19]

#### *3.2.5.2. Elution Chromatography*

Elution chromatography is characterized by the introduction of a discrete volume of the sample into the chromatographic column that has been previously equilibrated with the mobile phase. Typically, the volume of the sample is small compared to the volume of the column. The individual components of the sample (the solutes) move through the column at different average velocities, each less than the velocity of the mobile phase. The differences in velocities are caused by

differences in the interactions of the solutes with the stationary and mobile phases.[20]

#### *3.2.5.3. Displacement Chromatography*

Displacement chromatography is rarely, if ever, used for analytical separations, but it is useful for preparative separations. Many retentive chromatographic methods have been performed in the displacement mode, including normal-phase, reversed-phase, ion-exchange, and metal affinity chromatographies.

In the displacement technique, the sample is introduced to the column discontinuously and is displaced by a mobile phase, which interacts with the stationary phase more strongly than any component of the sample. Therefore, the displaced components leave the column before the mobile phase front.[10,21]

## 3.3. High Performance Liquid Chromatography

Nowadays, High Performance Liquid Chromatography (HPLC) is the most progressive analytical method available, with wide application in analyses of pharmaceutical preparations, cosmetics, foods, and beverages. Main advantages of HPLC are:

- HPLC is a separative method that enables quantitative and qualitative determination of compounds,
- analysis is very fast and highly sensitive,
- a minimal amount of a sample is needed,
- volatility of a sample is not necessary (in GC the volatility is the main condition).[8]

High performance liquid chromatography essentially is a highly improved form of classical column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced due to the action of a pump via high pressures of up to 40 MPa, which makes the chromatographic process much faster. The modern HPLC method also permits the use of a small particle size for the column packing material, which gives a much greater surface area for interactions between the stationary phase and the molecules

flowing past it. This allows much better separation of the components of the mixture.[22]

The chromatograph consists of components that ensure transport of the mobile phase, sample injection, separation process, and detection of compounds.[13]

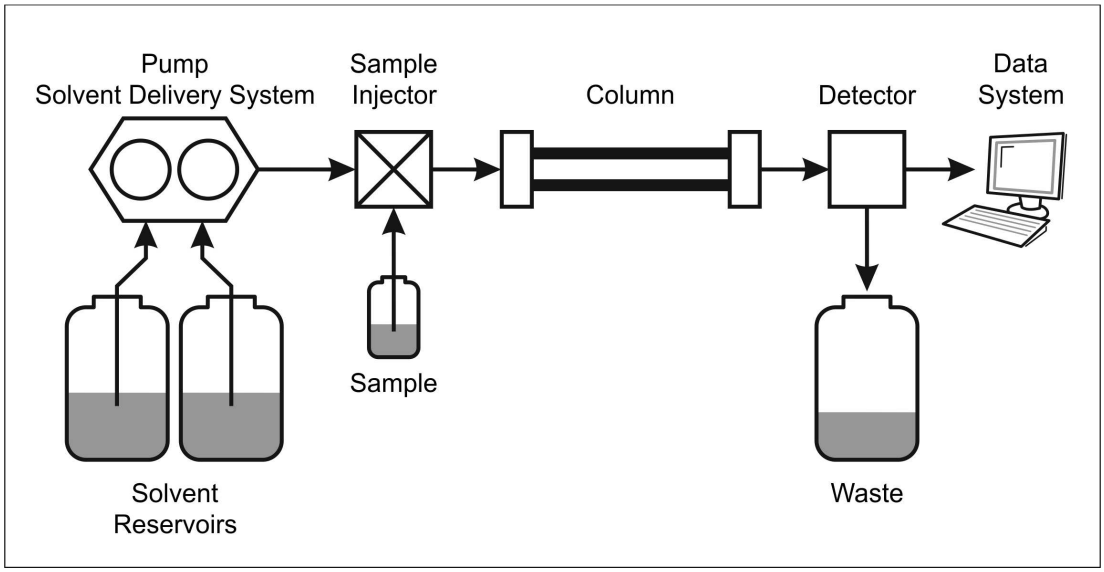


**Figure 1:** Scheme of the HPLC system. (Figure made by Jiří Hrůza)

The HPLC instrument includes a pump (solvent delivery system), a sample injector, a column, a detector and a data system. The heart of the system is the column where the separation is performed. Since the stationary phase is composed of micrometre sized porous particles, a high-pressure pump is required to move the mobile phase through the column. Separation of components occurs as the analytes and mobile phase are pumped through the column.[15]

### 3.3.1. Pump

High-pressure pumps are needed to force solvents through packed stationary phase beds. The pump must be a sturdy device capable of generating high pressures of up to 35 or even 50 MPa, because smaller bed particles require higher pressures. The range of flow usually goes from 0.1 ml $min^{-1}$ to 5 or 10 ml $min^{-1}$ and the flow rate should be pulseless.

The HPLC pumps can be divided into two categories: pulse pumps and pulsation-free pumps. The usual type of HPLC pump is the so-called short-stroke piston pump and it belongs to the group of pulse pumps. The mobile phase is driven by a piston whereas check valves open or close the path of the liquid towards the appropriate direction. With each stroke, the piston conveys a small amount of liquid, usually in the 100 µl range.

For very low solvent flows a pulsation-free pump can be used which does not convey the liquid by rapid strokes but rather acts like an oversized syringe. A slow-moving piston pushes the eluent directly from its reservoir but with this pump the gradients are impossible to perform.

For special conditions of separation, special two-piston pumps or pneumatic amplifier pumps are available.[13,15,16]

### 3.3.2. Sample Injector

Sample introduction can be accomplished in various ways. The simplest method is to use an injection valve. Two major designs exist: automatic injectors or manual injectors. In liquid chromatography, liquid samples may be injected directly and solid samples need only be dissolved in an appropriate solvent. It is always best to remove particles from the sample by filtering over a 5 µm filter, or centrifuging, since continuous injections of particulate material will eventually cause blockages in injection devices or columns.[15,23]

### 3.3.3. Column

The selective separation of a complex mixture into individual compounds by column liquid chromatography requires a sufficiently large surface of the adsorbent particles with interactive surface sites to selectively retain molecules based on their chemical structure and properties.

Typical HPLC columns are 50, 100, 150, and 250 mm length and they can be classified according to many criteria, e.g. according to the type of packing material, base material, etc.[15,24]

#### *3.3.3.1. Type of packing material*

A majority of packing materials used in HPLC are porous particles with average diameters between 1.8 μm and 10 μm. Porosity of the most commonly used silica-based packing materials employed as base materials for the separation of low-molecular weight analytes provides the surface area which is necessary for the analyte retention and which is usually between 100 and 400 $m^2/g$. Interparticle space is large enough (usually between 0.4 and 1.0 ml/g) to allow up to 1 – 3 ml $min^{-1}$ flow within acceptable pressure range. Within the pores the mobile phase (and the analytes) does not flow but moves only by diffusion.

If the stationary phase is nonporous then diffusion within pores does not occur. Particles with a diameter 1-2 μm are smaller and the capacity is about 50 times lower than compared to the porous particles. The column porosity is ca. 0.4 compared to 0.8 of column with porous packing. Chromatography of biopolymers seems to be more advantageous on these stationary phases with regard to denaturation and recovery.

As compared to particle bed columns, monolithic columns represent a single piece made of porous cross-linked polymer or porous silica. Monoliths are made in different formats as porous rods, generated in thin capillaries or made as thin membranes or disks. The diameter of the large pores (where the mobile phase flows through) is ca. 2 μm, the mean diameter of the skeleton structure is ca. 1.6 μm and the diameter of the mesopores is ca. 12 nm. Such materials have a porosity of more than 0.8, their separation performance similar to 3 μm porous particles.[7,16,24]

#### *3.3.3.2. Base material*

Interactions between the compounds of sample mixture and the stationary phase surface are the primary factor for successful separations.

Specific parameters of the base of packing material are:

- surface area,
- pore size,
- pore volume,
- pore size distribution,

- particle shape,
- particle size,
- particle size distribution,
- structural rigidity,
- chemical stability,
- surface reactivity,
- density and distribution of the surface reactive centers.

Surface area is directly related to the analyte retention. Generally, the higher the surface area, the greater the retention.

The most commonly used base material is silica ($SiO_2$). It consists of silicon atoms bridged three-dimensionally by oxygen atoms and the lattice is saturated at the surface with OH groups, the so-called silanol groups. One of the most important parameters is the metal content (content of sodium, calcium, magnesium, and aluminium) of the starting materials because this will determine the concentration of the acidic silanols. Silica may be chemically modified at these points to give phases with specific properties, i.e. chemically-bonded stationary phases. The silanol groups can be esterified, but esterified silica is prone to hydrolysis and so cannot be used with mobile phases containing water or alcohol. Other type of modification is Si-N bond so these products have better hydrolytic stability. The most stable of all, the octadecylsilane [$–(CH_2)_{17}CH_3$) ] is the most widely used of these chemically modified products. It is extremely nonpolar and is the preferred choice for use in reversed-phase chromatography.

Non-modified silica has one significant drawback: it is soluble at high pH, although chemical modification with high bonding density of attached alkylsilanes extends its stability range to over pH 10.

Another porous base material suggested as an alternative to silica is zirconia. Zirconia is stable in very wide pH range (pH 1-14), but zirconia's surface has relatively low reactivity (more difficult to bond different functional groups to the surface), which significantly limits a selection of available stationary phases.

Polymer-based materials have been on the market for more than 30 years. Cross-linked styrene-divinylbenzene and methylmethacrylate copolymers are the most widely used. These materials show high pH stability and chemical

inertness. Their rigidity and resistance to the swelling in different mobile phases is independent on the degree of crosslinkage.

Some other stationary phases are: aluminia, magnesium salicate, controlled-pore glass, methacrylate gels, hydroxylapatite, agarose, porous graphitic carbon and titania.[7,16]

### 3.3.4. Detector

Since the development of HPLC as a separation technique, considerable effort has been spent on the design and improvement of suitable detectors. The detector is perhaps the second-most important component of an HPLC system, after the column that performs the actual separation.

Over the years, many physical techniques, from nuclear magnetic resonance (NMR) to electrochemistry, have been applied to HPLC detection, and a number of them have become widely used. An analytical laboratory is virtually certain to need several different types of detectors, as no single modality will be suited to the range of analytes, sample volumes, and separation methods used. Hybrid detectors that combine two or more modes in a single instrument are becoming more popular, as they can extract more information from each sample run. There are several major characteristics to consider when comparing different chromatographic detectors. The main concerns are usually sensitivity, noise, and dynamic range, which together determine the minimum and the maximum detectable amount of analyte, but other features can also require careful matching to the desired application. A highly selective detector will respond to relatively few analytes; this can be extremely useful in identifying a few compounds of interest in a complex mixture, but in other applications could lead to missed peaks.[25]

#### *3.3.4.1. UV-VIS Detector*

The use of a fixed wavelength UV detector for LC separations was first described by Horvath and Lipsky in 1966, and is possibly the most popular HPLC detector in general use today.[26]

Although other detection techniques are more sensitive, the UV detector provides simple and universal answer to the majority of HPLC applications.[27]

The main principle of the UV-VIS detector is described as follows: The fraction of light transmitted through the detector cell is related to the solute concentration according to Lambert-Beer's Law. The UV-Vis detector has its advantages: it is specific and highly sensitive (from $10^{-9}$ to $10^{-10}$ g/ml); it has good stability and is capable to use gradient elution.[8,23]

The fixed wavelength UV detector uses light of a single wavelength (or nearly so) which is produced by a specific type of discharge lamp. The most popular lamp is the low pressure mercury vapor lamp, which generates most of its light at a wavelength of 254 nm. Other lamps that could be used are the low-pressure cadmium lamp and low pressure zinc lamp. None of the lamps are strictly monochromatic and light of other wavelengths is always present but usually at a significantly lower intensity.[28]

The multi–wavelength detector employs a light source that emits light over a wide range of wavelengths. Employing an appropriate optical system, light of a specific wavelength can be selected for detection purposes. Light of a specific wavelength might be chosen where a solute has an absorption maximum to provide maximum sensitivity.[29]

Compared to conventional UV detectors, the diode array detector (DAD) is built with inverse optics. The light first passes a detector cell, and then is divided spectrally in a polychromator. Finally, the spectral light reaches the diode array, i.e. chip with a large number (100 – 1000) of light-sensitive diodes that are arranged side by side. Each diode only obtains a well-defined fraction of the information which is read by the computer.[16,30]

This type of detector is able to online measure UV spectra within the range from 190 to 950 nm and wavelength resolution is up to 1 nm.[23]

#### *3.3.4.2. Refractive Index Detector*

The refractive index detector (RI detector) is a commonly used universal detector that measures a physical property of the bulk medium. RI is the physical parameter that characterizes the velocity of light in a medium and is used to describe the behavior of light as it passes between different materials. The angle of deflection, which changes as the analyte peaks pass through the sample chamber, is recorded by the RI detector.

There are two major limitations: first, RI detectors are very sensitive to changes in the temperature, pressure, and flow rate of the mobile phase, and so these measurement conditions must be kept stable in order to obtain low background levels. Second, RI detectors are incompatible with chromatographic separations using gradient elution. Furthermore, because RI detectors are nonselective, they must be used in conjunction with other detection methods if specificity is required. Nevertheless, they have found wide application in isocratic chromatographic analysis for analytes that do not have absorptive, fluorescent, or ionic properties. Sensitivity of this detector is $10^{-6}$ g/ml.[8,25]

#### *3.3.4.3. Fluorescence detector*

There are several kinds of luminescence, or processes in which light is emitted from a material. The emitted light can arise from chemical reactions, as in chemiluminiscence, or mechanical crushing or rubbing, as in triboluminiscence. Fluorescence is a type of photoluminescence in which material excited by a light at one wavelength, or a range of wavelengths, emits light at a longer wavelength. Fluorescence detection is also extremely selective, as strong fluorescence from excitation with visible light is relatively uncommon in natural materials. If required, the fluorescent derivative of the substance of interest may be prepared (employing an appropriate fluorescent reagent).[25,31]

Typically, sensitivity of fluorescence detector (FLD) is 10 – 1000 times higher (from $10^{-9}$ to $10^{-12}$ g/ml) than sensitivity of the UV detector for strong UV absorbing materials. Fluorescence detectors are very specific and selective among the others optical detectors.[8,16]

#### *3.3.4.4. Conductivity detector*

Conductivity detector can be used whenever the sample bands have different conductivity from the running buffer, although historically they have been used most extensively for the detection of small inorganic ions. The resistance depends of the effluent, and particularly on the concentration and mobilities of the ionic species.

There are two main varieties of bulk conductivity detectors: contact and contactless. In a contact conductivity detector, the electrodes contact the column

effluent directly. The electrodes are usually made of stainless steel, platinum, or gold in order to minimize electrochemical reactions, but they are still subject to fouling over time. In a contactless conductivity detector, the electrodes are separated from the column effluent by insulating barriers, and are capacitively coupled to the sample. Because the electrodes are isolated, there is no possibility of fouling, so the contactless detection cells tend to be more robust.[25]

#### *3.3.4.5. Electrochemical detector*

Electrochemical detector belongs to the group of the most sensitive detectors used in separation science and is applicable to drug analysis, clinic chemistry and neuroscience. The electrochemical detector measures the electron flow that is generated at electrode surface during oxidation or reduction reactions.

There are two types of detectors: coulometric detectors and amperometric detectors. In coulometric detection the reaction goes to completion, the total charge generated is proportional to the total mass of the reactant. In amperometric detection the mobile phase will flow the reactant over the electrode and produce a current that varies with the concentration of the analyte being measured. Sensitivity of this detector is from $10^{-9}$ to $10^{-12}$ g/ml.

Most electrochemical detectors require three electrodes: the working electrode, the auxiliary electrode, and the reference electrode. The working electrode is where oxidative or reductive activity takes place, whole the auxiliary and reference electrodes compensate for charges in the mobile phase conductivity. In all cases, the electrodes are controlled by simple circuitry involving a regulated power supply, a potentiometer, and a series of amplifiers.[8,25]

#### *3.3.4.6. Radiochemical detector*

Radiochemical detectors used in HPLC are based on principle of energy emitted from either ß particles (tritium and carbon-14) or low-energy γ-ray sources (iodine-125) that can be absorbed by the scintillators, which transform the energy into light in the range of 350 – 600 nm. This light is detected and its intensity is proportional to the energy of the radionucleotide emission.

Radiochemical detection often suffers from reduced sensitivity due to high background.[25]

#### *3.3.4.7. Evaporative Light Scattering detector*

The evaporative light scattering detector (ELSD) is considered as a universal detection technique; the only requirement is that the sample compounds must be less volatile than all the components of the mobile phase.

The ELSD includes three distinct stages that need to be optimized in order to achieve low background, high sensitivity, and repeatability. These are the nebulization, evaporation, and optical detection stages. Therefore, the ELSD is a destructive method, so no further analysis of the sample peaks is possible.

The ELSD is a universal detector, allowing direct detection of compounds, such as carbohydrates that are not amenable to UV absorption, fluorescence or electrochemical detection without derivatization.[25]

#### *3.3.4.8. Liquid Chromatography – Mass Spectrometry*

The power of mass spectrometry lies in the fact that the mass spectra of many compounds are sufficiently specific to allow their identification with a high degree of confidence, if not with complete certainty. If the compound of interest is encountered as part of a mixture the mass spectrum obtained will contain ions from all of the compounds present. The combination of the separation capability of chromatography to allow 'pure' compounds to be introduced into the mass spectrometer with the identification capability of the mass spectrometer is clearly therefore advantageous, particularly as many compounds with similar or identical retention characteristics have quite different mass spectra and can therefore be differentiated. This extra specificity allows quantitation to be carried out which, with chromatography alone, would not be possible. Thus, the combination of HPLC with mass spectrometry allows more definitive identification and the quantitative determination of compounds that are not fully resolved chromatographically.

A wide range of molecules, from low-molecular-weight drugs, and metabolites (<1000 Da) to high-molecular-weight biopolymers (>100 000 Da), may be studied by HLPC combined with mass spectrometry.[32]

## 3.4. System Suitability Test

Before performing any validation experiments, the analytical chemist should establish that the HPLC system and procedure are capable of providing data of acceptable quality. These tests are used for verification that the resolution and efficiency of the system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronics, analytical operations, and samples constitute an integral system that can be evaluated as a whole. System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns. The system suitability test represents an integral part of the method and is used to ensure the adequate performance of the chosen chromatographic system.[1,33]

Efficiency, symmetry factor, and resolution are the parameters that are normally used in assessing the column performance.[9]

### 3.4.1. Efficiency

The efficiency is the measure of the chromatographic band broadening and the number of the theoretical plates ($N$) in the column. Each plate has a finite height (height of the effective theoretical plate), and an analyzed compound spends a finite time in this plate. This time is considered to be sufficient to achieve equilibrium. The plate height is smaller or the number of the plate is greater, the analyte exchange is more efficient between two phases and the separation is better.

The efficiency is the property of the column and is stated by producer of the column. The efficiency can be calculated using the following equation:

$$N = 5{,}545 * \left(\frac{t_R}{w_h}\right)^2$$

where $t_R$ is the retention time or the baseline distance between the point of injection and the perpendicular dropped from the maximum of the peak of interest, and $w_h$ is the width of the peak of interest determined at half peak height, measured in the same units as $t_R$.[7,9,34,35]

### 3.4.2. Symmetry factor

The symmetry parameter describes the symmetry aspects of the peak appearance. If a value of $A_s$ is approximately 1, the peak is practically symmetric. Values of $A_s$ which are greater than 2 may lead to incorrect integration, resulting in erroneous quantitation. The main factors that influence peak symmetry depend upon retention, solvent effects, incompatibility of the components with the mobile phase.

The symmetry factor for a peak can be calculated by the following equation:

$$A_s = \frac{\mathrm{w}_{0.05}}{2 * \mathrm{d}}$$

where $w_{0.05}$ is the peak width at 5% of peak height, measured from the baseline, and $d$ is the baseline distance between the perpendicular dropped from the peak maximum, and the leading edge of the peak at 5% of the peak height, measured in the same units as $w_{0,05}$.[9,34,35]

The symmetry parameter $A_s$ should be in interval from 0.8 to 1.5.[34,35]

### 3.4.3. Resolution

The distance between the peak maxima reflects the selectivity of the system. The greater the distance, the higher the selectivity. The resolution is defined as the ratio of the distance between two peaks to the widths of these peaks (at half peak height).

The resolution of two peaks of similar height in a chromatogram can be calculated using the following equation:

$$R_s = \frac{1{,}18 * (t_{R2} - t_{R1})}{w_{h1} + w_{h2}}, t_{R2} > t_{R1}$$

where $t_{R1}$ and $t_{R2}$ are retention times or baseline distances between the point of injection and the perpendicular dropped from the maximum of each of the two peaks, and $w_{h1}$ and $w_{h2}$ are the respective peak widths determined at half peak height, measured in the same units as $t_{R1}$ and $t_{R2}$.[7,9,34,35]

The value of $R_s$ for the baseline separation of the peaks of similar height should be at least 1.500.[34,35]

## 3.5. Chromatographic method validation

The Food and Drug Administration in publication called *Guidance for Industry, Analytical Procedures and Methods Validation* (2000), states:

> **'Methods validation is the process of demonstrating that analytical procedures are suitable for their intended use. The methods validation process for analytical procedures begins with the planned and systematic collection by the applicant of the validation data to support the analytical procedures.'**[36]

Analytical method validation is established through documented evidence demonstrating the accuracy, precision, linearity, selectivity, ruggedness, and/or robustness of that particular test method which will be utilized to generate test results for a drug substance or drug product. Different test methods require different validation parameters. All analytical procedures require some form of method validation, regardless of whether the test method is utilized for testing Good Laboratory Practice (GLP) toxicology, in-process controls, clinical release or others.[7]

### 3.5.1. Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.[1]

During the validation, accuracy is determined by measuring the recovery of the active component from a drug product matrix or by directly measuring the active pharmaceutical ingredient (API). Typically studies involve spiking the drug product placebo matrix with API in amounts equal to the nominal finished dosage strength.[37]

Method accuracy should be reported as percent recovery which can be calculated by the following equation:

$$R(\%) = 100 * \frac{c_i}{c_0}$$

where *R (%)* is recovery, $c_0$ is concentration real (added), and $c_i$ is concentration determined (measured).

### 3.5.2. Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple samplings of the same homogeneous sample under the prescribed conditions.[1]

Validation of the HPLC method precision can be subdivided into three stages. The first stage is injection precision (injection repeatability) based on multiple injections of a single preparation of a sample on a given day. The second stage is method precision (analysis repeatability) where multiple preparations and multiple injections of a sample are analyzed by the same chemist on the same day. The third stage is intermediate precision and is usually performed by different analysts, on a different system, on a different day, on the same drug substance or drug product batch to determine the variability of the analytical test. The intermediate precision may give indications to potential issues that may arise during method transfer.[7]

### 3.5.3. Specificity and Selectivity

The specificity is the ability to assess unequivocally the desired component in the presence of components that may be expected to be present such as impurities, degradation products, and excipients. There must be unarguable data for a method to be specific. Specificity measures only the desired component without interference from other species which might be present. Separation is not required.[1,37]

The term selectivity is sometimes used interchangeably with specificity. Technically there is a difference. The selectivity is defined as the ability of the method to separate the analyte from other components that may be present in the sample (desired or not desired), including impurities. No chromatographic peaks, such as related compounds, should interfere with the desired compound

peak. Separation is required. Therefore, a method that is specific may not be selective [37]

### 3.5.4. Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.[1]

If the method is linear, the test results are directly or by well-defined mathematical transformation proportional to the concentration of analyte in samples within a given range. Linearity is usually expressed as the confidence interval around the slope of the regression line. The line is calculated according to an established mathematical relationship from the test response obtained by the analysis of samples with varying concentrations of analyte.

During validation, linearity may be established for all active substances, preservatives, and expected impurities. Evaluation is usually performed on standards.[37]

## 3.6. Evaluation

### 3.6.1. Qualitative - Identification

For identification of an analyzed compound two parameters are usually obtained: the maximum of absorption and the retention time.

#### *3.6.1.1. Maximum of Absorption*

For the most precise and accurate evaluation of samples, it is necessary to obtain information about maxima of absorption ($\lambda_{max}$) of specific analyzed compound. Firstly, from UV spectrum it is needed to select the wavelength in which specific analyzed compound has the highest possible (maximum of absorption) or sufficiently applicable absorption. This wavelength is used for all other experiments.

Secondly, for specific identification, the parameters obtained from UV spectra of a sample of analyzed compound need to be compared with the parameters from UV spectra of a standard of same analyzed compound. These parameters are e.g. maxima and minima of absorption. It is required to use the same chromatographic system conditions for both measurements (e.g., conditions published in Pharmacopoeias).[8]

#### *3.6.1.2. Retention time*

The distance of the peak maxima from the injection point expressed in time units is called retention time ($t_R$), and it serves as an qualitative identifier for the given analyte on that particular system. Retention time is probably the most widely used descriptor of the analyte behaviour, and it is the most easily measurable parameter. Analyte retention time is dependent on the mobile phase flow rate, and flow rate stability.[7]

### 3.6.2. Quantitative - Assay

To determine exact content of analyzed compounds in a sample it was essential to compare parameters (peak areas or height of peaks) of a standard (known content of compounds) with a sample (determined content

of compounds). For calculation of exact content the internal standard method or the external standard method are usually used.

#### *3.6.2.1. Internal Standard Method*

Internal standards are especially useful for analyses in which the quantity of sample components or the instrument response varies slightly from run to run for reasons that are difficult to control.[38]

Internal standard is an inert compound added to a sample in known concentration to facilitate the quantitative determination of the sample components. Because both components (analyzed compound and internal standard) are present in same sample mixture, errors from external influences are thus eliminated.[6,39]

#### *3.6.2.2. External Standard Method*

The use of an external standard procedure is probably the simplest methodology that may be employed. External standard is a compound present in a standard sample of known concentration and volume which is analyzed separately from the unknown sample under identical conditions. It is used to facilitate the quantitative determination of the sample components. It is necessary to perform HPLC method under strictly identical conditions.[6,32]

The concentration of an analyzed compound can be calculated by following equation:

$$c_{\%} = \frac{A_x * m_{St} * F * 100}{A_{St} * m_x * Z}$$

where $c_{\%}$ is a concentration of the analyzed compound in %, $A_x$ and $A_{St}$ are peak areas of the sample ($x$) and the standard ($S$), $m_x$ and $m_{St}$ are weighed amounts of the sample ($x$) and the standard ($S$), $F$ is the response factor of the standard and $Z$ is the dilution factor.

## 3.7. Charasteristics

### 3.7.1. Dexpanthenol

Pharmacopoeial name: **Dexpanthenolum**

Chemical name:

(*R*)-(+)-2,4-dihydroxy-N-(3-hydroxypropyl)-3,3-dimethylbutyramide[34]

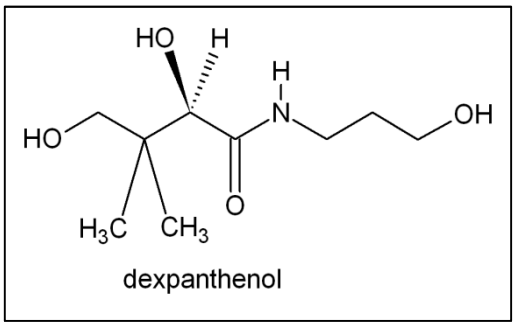


**Figure 2:** Chemical structure of dexpanthenol.

**Characters**: A colourless or slightly yellowish, viscous hygroscopic liquid, or a white or almost white, crystalline powder, very soluble in water, freely soluble in ethanol (96 %).[34]

Dexpanthenol (provitamin $B_5$) is the right isomer of panthenol and has equal biological activity as its metabolite pantothenic acid (vitamin $B_5$). Dexpanthenol is also more stable than its metabolite, especially in aqueous solutions. Vitamin $B_5$ is necessary for synthesis of coenzyme A, which is known to have an important role in the metabolism of carbohydrates, fats and nitrogen compounds.[40]

Dexpanthenol is individually therapeutically administered to maintain functionality of mucus and skin cells, simultaneously applied with treatment of colon amyotonia and neuropathy. Dexpanthenol is also used as supportive treatment of mucus affects, such as inflammation of upper respiratory tract (catarrh of nasal mucus, bronchitis, tracheitis, laryngitis, pharyngitis, stomatitis, etc.). Various pharmaceutical formulations in concentrations 3 – 5% are used externally to increase epithelializathion of burn and scratch wounds as

well as ulcers. Dexpanthenol is usually formulated in multi-vitamin preparations, parenterals, and some local cosmetic preparations.[2,40]

Several papers have been published about determination of dexpanthenol or pantothenic acid in multi-vitamin preparations or local formulations.

- Herminia Umagat et al. developed a method of determination of panthenol in bulk, premix and liquid multivitamin preparations. Aminopropanol, which is formed by the hydrolysis of panthenol, is reactivated with fluorescamine, a reagent specific for primary amines. They used Chromegabond $C_{18}$ as a stationary phase (300 mm × 4.6 mm I.D.), and mobile phase containing 300 ml of methanol and 700 ml of 0.1 M borate buffer, pH adjusted to pH 8.0 with 2 M sodium hydroxide. UV-VIS detector was used.[41]

- Bořivoj Klejdus et al. analyzed dexpanthenol in various pharmaceutical preparations as one of number of water- and fat-soluble vitamins. The separation was achieved by using MetaChem Polaris column $C_{18}$A (150 mm × 4.5 mm I.D.) and combined isocratic and linear gradient elution with mobile phase consisting of 0.01 % trifluoroacetic acid of pH 3.9 and methanol. The detection wavelength is 280 nm.[42]

- Pilar Vinas et al. developed a reversed-phase method for the determination of the B group vitamins in baby foods using analytical column packed with RP-Amide$C_{16}$ and combined mobile phase in gradient mode. The detector was a photodiode array Shimadzu operating at four wavelengths: 249 nm, 266 nm, 326 nm and 361 nm.[43]

- M. Y. Xie et al. described a validated LC method for determination of enantiomeric purity of panthenol in bulk drugs. They derivatized panthenol with 3,5-dinitrobenzoyl chloride. The stationary phase was Kromasil CHI-DMB (250 × 4.6 mm I.D.) and as the complex mobile phase n-hexane and ethanol (95:5, v/v) was used. The detection was performed at 265 nm.[44]

- Jin Pengfei et al. analyzed complex of B-vitamins and C-vitamin in multivitamin preparation. They used Alltima $C_{18}$ column (250 mm × 4.6 mm I.D.) as the stationary phase and 50 mM ammonium dihydrogen phosphate (adjusting with phosphoric acid to pH 3.0) and acetonitrile as the mobile phase. Pantothenic acid was extracted together with $B_1$ and $B_3$ vitamins by a solution containing 0.05 % phosphoric acid (v/v) and 0.3 sodium thiosulfate (w/v) and simultaneously analyzed by the mobile phase containing phosphate buffer and acetonitrile (95:5, v/v). Detection wavelength was 210 nm for pantothenic acid. [45]

- Stojanka Vidović et al. developed HPLC stability-indicating method for simultaneous determination of some water-soluble vitamins and preservatives in multivitamin syrup preparation. As the stationary phase Zorbax SB-Aq $C_{18}$ (250 mm × 4.6 mm I.D.) was used. Combined isocratic and gradient elution was performed with a mobile phase consisting of 0.0125 M hexane-1-sulfonic acid sodium salt in 0.1 % (m/v) *o*-phosphoric acid, pH 2.4 – 2.5 (solvent A) and acetonitrile (solvent B). Detection wavelength for pantothenic acid was 210 nm.[46]

- Mao X. et al. presented validated method for simultaneous determination of pantothenic acid and dexpanthenol in cosmetics with different matrices.[47]

- A. U. Kulikov et al. developed a isocratic HPLC method for the assay of dexpanthenol in aerosol and gel. As the stationary the Vydac Proteins C4 column was used and the mobile phase was aqueous solution of trifluoreacetic acid. The UV detection at 206 nm was used.[40]

Other papers describe methods of determination of pantothenic acid by other methods.[48,49]

### 3.7.2. Preservatives

- Pharm. Name: **Methylparabenum,** Methylis parahydroxybenzoas
  Chemical name: Methyl *p*-hydroxybenzoate
- Pharm. Name: **Ethylparabenum,** Ethylis parahydroxybenzoas
  Chemical name: Ethyl *p*-hydroxybenzoate
- Pharm. Name: **Propylparabenum,** Propylis parahydroxybenzoas
  Chemical name: Propyl *p*-hydroxybenzoate
- Pharm. Name: **Butylparabenum,** Butylis parahydroxybenzoas
  Chemical name: Butyl *p*-hydroxybenzoate
- **Isobutylparabenum,** synonym: Isobutylis parahydroxybenzoas
  Chemical name: Isobutyl *p*-hydroxybenzoate
- Pharm. Name: **Phenoxyethanolum**
  Chemical name: 2-Phenoxyethan-1-ol[34]

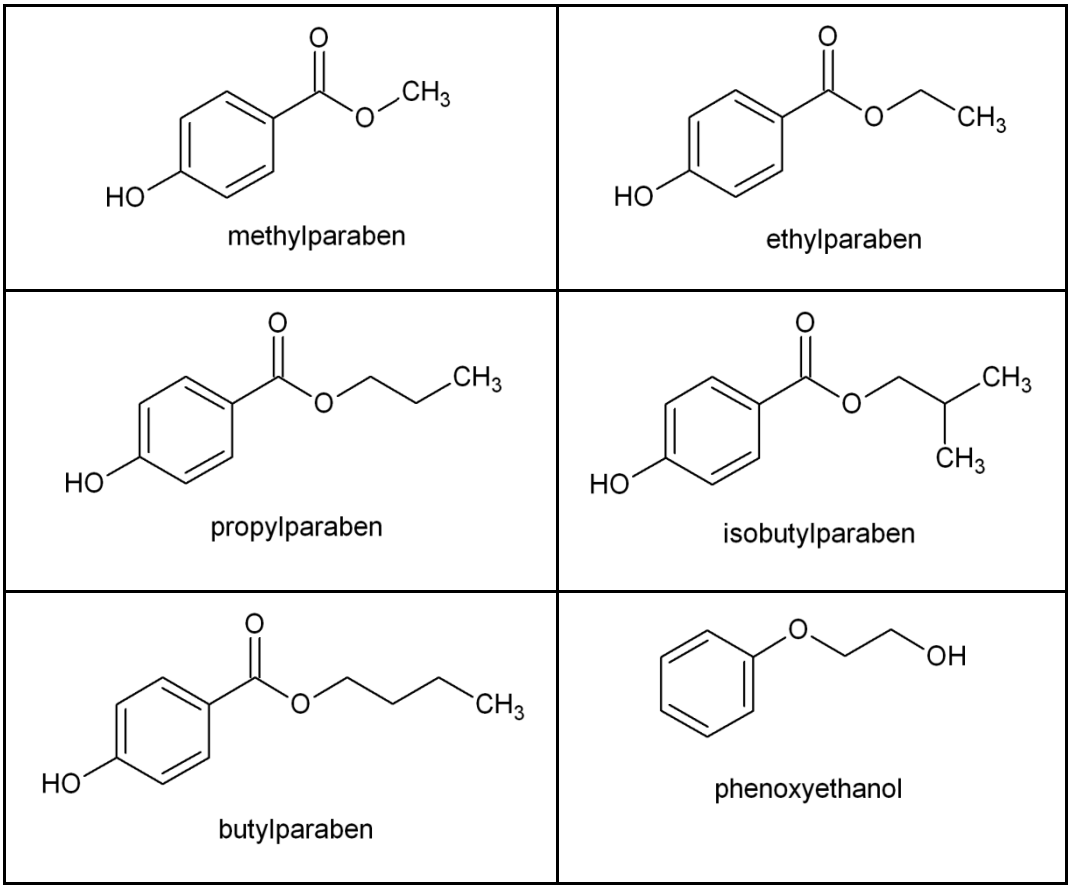


**Figure 3:** Chemical structures of preservatives and phenoxyethanol.

**Characters:**

- Parabens: colourless crystals or a white/almost white crystalline powder; very slightly soluble in water; soluble in boiling water; freely soluble in ethanol (96 %), and methanol.
- Phenoxyethanol: colourless, slightly viscous liquid; slightly soluble in water, miscible with acetone, with ethanol (96 %), and with glycerol, slightly soluble in arachis oil, and in olive oil.[34]

Parabens are a homologous group of esters of 4-hydroxybenzoic acid commonly used as antimicrobial preservatives in pharmaceutical formulations, foods, beverages, and cosmetics. Methylparaben (in concentration range 0.015 – 0.3 %), ethylparaben, propylparaben (in concentration range 0.005 – 0.6 %), butylparaben (in concentration range 0.06 – 0.4 %), and isobutylparaben are often used in pharmaceutical preparations applicable in the most of administration ways but primarily for dermal and oral use. With prolonging the side alkyl chain of ester group the solubility in aqueous solutions descends and antimicrobial effect increases.

Phenoxyethanol is derivate of ethanol employed as solvent for preservatives. Nevertheless, phenoxyethanol can be also used as potentiating antimicrobial preservative added to increase antimicrobial effect of other preservatives.[50,51]

Several papers have been published about determination of preservatives individually or in the present of other compounds.

- Anastasia Zotou et al. presented a method for separation and determination of five parabens in saliva and toothpaste by HPLC with UV detection. As the stationary phase a short monolithic column (50 mm × 4.6 mm I.D.) was used and gradient elution by $H_2O$ and acetonitrile was used as the mobile phase. The detection wavelenght was 254 nm. Phenoxyethanol was used as internal standard.[3]

- Wenhui Gao et al. developed a validated analytical method for determination of hydroquinone, phenol and six preservatives in cosmetic creams. As the statinosary phase Zorbax Bonus-RP $C_{18}$ column (100 mm

× 2.1 mm I.D., 3.5 μm) was used and the mobile phase consisted of methanol and 0.05 mol/l ammonium formate in water (pH = 3.0) with gradient elution. The detection was made at 280 nm.[52]

- M. Jamil Akhtar et al. described a reverse-phase liquid chromatographic technique for determination of phenoxetol, methylparaben, ethylparaben, n-propylparaben, isobutylparaben, n-butylparaben and croconazole hydrochloride. L-Bonda Pak $C_{18}$ (300 mm × 3.9 mm I.D.) columns were used as the stationary phases, protected by an octadecyl silane pre-column. The isocratic elution of 0.05 M potassium dihydrogen phosphate (pH 3.50) and acetonitrile (65:35, v/v). The detection was performed at 254 nm.[53]

Other papers describe methods of determination of preservatives by other methods, e.g. with chemiluminiscence detection, gas chromatography, etc.[54–56]

# Chapter 4 Experimental part

## 4.1. Equipment

**High Performance Liquid Chromatography:**

- High Performance Liquid Chromatography System, Agilent Technologies Infinity 1290, Agilent Technologies, CA, USA
    - Binary Pump G4220A, Serial No. DEBAA01465
    - Thermostat G1330B, Serial No. DEBAK05496
    - Autosampler G4226A, Serial No. DEBAP01797
    - Thermostated Column Compartment G1316C, Serial No. DEBAC02344
    - Diode-Array Detector G4212B, Serial No. DEAA301966
    - Fluorescence Detector G1321B, Serial No. DEABO01345

**Columns:**

- Column Discovery $C_{18}$, 150 mm × 4.6 mm I.D., particle size 5 µm, Serial No. 124974-01, bonded-phase lot. 7521, Supelco Analytical, Sigma Aldrich, MO, USA
- Column ZORBAX Eclipse Plus $C_{18}$ Rapid Resolution HD, 50 mm × 2.1 mm I.D., particle size 1.8 µm, Serial No. U3DAY06202, Agilent Technologies, CA, USA

**Ultrasonic bath:**

- Ultrasonic bath Bandelin SONOREX RK 100, Serial No. 301.00063238.001, Berlin, Germany

**Balance:**

- Analytical Balance Mettler Toledo XP105DR/M, Serial No. 139297660, Mettler Toledo, Switzerland
- Balance Mettler Toledo PL202-L, Serial No. B14234570, Mettler Toledo, Switzerland

**Filters:**

- Syringe filter 0,45 µm F2600-1, Lot No. 00135343, OEM Syringe Filters
- Membrane filters for the mobile phase - Nylon membrane 66 MNY 4547, 0,45 µm × 47 mm, Supelco Analytical, Sigma Aldrich, MO, USA

**Pipette:**

- Pipette 100 – 1000 µl, Fisher Scientific, MA, USA

**pH meter:**

- SevenCompact pH/Ion S220 Mettler Toledo AG, Serial No. 12323154476, Mettler Toledo, Switzerland

**Software:**

- Open LAB CDS ChemStation Edition, Agilent Technologies, CA, USA
- EffiValidation 3.0, EffiChem, Czech Republic

**Glassware:**

- Calibrated glassware was used.

## 4.2. Materials

### 4.2.1. Standards

**Table 1:** List of the standards.

| Name | Product code | Producer | Batch No. | Purity |
|---|---|---|---|---|
| Dexpanthenol | 77624 | Sigma Aldrich | BCBD4354 | Ph Eur grade |
| Methylparaben | 54750 | Sigma Aldrich | BCBC4082V | Puriss., ≥ 99.9% (GC) |
| Ethylparaben | 111988 | Sigma Aldrich | STBB6758V | ReagentPlus®, 99% |
| Propylparaben | P53357 | Sigma Aldrich | STBB3709V | ≥ 99% |
| Butylparaben | 54680 | Sigma Aldrich | BCBD7360V | ≥ 99.0% (GC) |
| Isobutylparaben | 715077 | Sigma Aldrich | MKBH7398V | 97% |
| Phenoxyethanol | 77699 | Sigma Aldrich | BCBF2337V | ≥ 99% |

### 4.2.2. Chemicals

**Table 2:** List of the chemicals.

| Name | Product code | Producer | Batch No. | Purity |
|---|---|---|---|---|
| Acetonitrile for HPLC | 34851 | Sigma Aldrich | SZBB120SV | Chromasolv ® gradient grade ≥ 99.9 % |
| Aqua for HPLC | 34877 | Sigma Aldrich | BCBG2188V | Chromasolv ® Plus |
| Methanol for HPLC | 34860 | Sigma Aldrich | SZBB3530V | Chromasolv ®, 99.9 % |
| Potassium dihydrogen phosphate | 60220 | Sigma Aldrich | SZBA3580V | Buffer substance, anhydrous, puriss. p.a., ASC reagent, reag. ISO, Ph. Eur., 99.5-100.5% |
| Phosphoric acid 85% | 30417 | Sigma Aldrich | SZBB0470V | Puriss. p.a., ASC reagent, reag. ISO, Ph. Eur., ≥ 85% |

### 4.2.3. Samples

**Table 3:** List of the samples.

| Name of sample | Batch No. |
|---|---|
| *Panthenol 4% gel** | 329/2011 |
| *Placebo Panthenol 4% gel without panthenol*** | 330/2011 |
| *Placebo Panthenol 4% gel without panthenol and preservatives**** | 333/2011 |

* *Panthenol 4% gel* is the special testing batch of topic gel based on the acrylamide polymer, containing the dexpanthenol, the phenoxyethanol, and the preservatives, i.e. the methylparaben, the ethylparaben, the propylparaben, the butylparaben and the isobutylparaben.

** *Panthenol 4% gel without panthenol* is the special testing batch of topic gel based on the acrylamide polymer, containing the phenoxyethanol and the preservatives.

*** *Panthenol 4% gel without panthenol and preservatives* is the special testing batch of topic gel containing only the acrylamide polymer.

## 4.3. Preparations

### 4.3.1. Preparation of Standards

- ***Solution of the parabens.***

*The solution of the parabens* was prepared by weighing 80 mg of the methylparaben, 20 mg of the ethylparaben, 10 mg of the propylparaben, 20 mg of the butylparaben, and 10 mg of the isobutylparaben. Standards were dissolved in 25 ml of acetonitrile in a 25 ml volumetric flask.

- ***Working standard solution A* and *Working standard solution B.***

The working standard solutions were prepared by weighing 100.0 mg of the dexpanthenol and 14.4 mg of the phenoxyethanol and dissolving in a small amount of acetonitrile in a 100 ml volumetric flask. From *the Solution of the parabens,* 1.00 ml was measured by pipette and placed into the volumetric flask with the dexpanthenol and the phenoxyethanol standards. The remaining acetonitrile was added into the flask to a volume of 100 ml.

**Table 4:** List of compounds in *the working standard solution A* and *the working standard solution B*.

| Compound | Concentration | |
|---|---|---|
| | **Working standard solution A** | **Working standard solution B** |
| **Dexpanthenol** | 99.52 mg/100 ml | 100.91 mg/100 ml |
| **Phenoxyethanol** | 14.23 mg/100 ml | 14.12 mg/100 ml |
| **Methylparaben** | 3.194 mg/100 ml | 3.1908 mg/100 ml |
| **Ethylparaben** | 0.810 mg/100 ml | 0.792 mg/100 ml |
| **Propylparaben** | 0.408 mg/100 ml | 0.4084 mg/100 ml |
| **Isobutylparaben** | 0.390 mg/100 ml | 0.4128 mg/100 ml |
| **Butylparaben** | 0.814 mg/100 ml | 0.7968 mg/100 ml |

### 4.3.2. Preparation of Mobile Phases

- **Potassium dihydrogen phosphate buffer (0.01mol $l^{-1}$).**

The buffer solution was prepared by weighing 1.36 g of potassium dihydrogen phosphate and dissolving it into 1000 ml of Aqua for HPLC, pH adjusted to 2.50 with the phosphoric acid 85%.

- **Methanol.**

Only methanol for HPLC was used.

- **Acetonitrile.**

Only acetonitrile for HPLC was used.

### 4.3.3. Preparation of Samples

- ***Panthenol 4% gel sample.***

*The Panthenol 4% gel sample* was prepared by weighing 0.5 g of the Panthenol 4% gel (equivalent to 0.02 g of the dexpanthenol) into the centrifugal test tube with 20.0 ml of acetonitrile. The sample was covered with parafilm and placed into the ultrasonic bath for 10 minutes. The sample was then centrifugated for 15 minutes at 4500 rpm. The supernatant (2 ml) was filtered into a HPLC vial using a nylon syringe filter.

- ***Placebo Panthenol 4% gel without dexpanthenol sample.***

*The Placebo Panthenol 4% gel without dexpanthenol sample* was prepared by weighing 0.5 g of the Panthenol 4% gel without dexpanthenol into the centrifugal test tube with 20.0 ml of acetonitrile. The rest of the preparation was identical with *the Panthenol 4% gel sample* preparation.

- ***Placebo Panthenol 4% gel without dexpanthenol and preservatives sample.***

*The Placebo Panthenol 4% gel without dexpanthenol and preservatives sample* was prepared by weighing 0.5 g of the Panthenol 4% gel without dexpanthenol and preservatives into the centrifugal test tube with 20.0 ml of acetonitrile. The

rest of the preparation was identical with *the Panthenol 4% gel sample* preparation.

### 4.3.4. Preparation of Validation Studies Solutions

- ***Accuracy studies solutions.***

To evaluate the accuracy of the method, recovery studies of all compounds were carried out by the addition of *the working standard solution A* to *the Placebo Panthenol 4% gel without dexpanthenol and preservatives sample* at three different concentration levels (80%, 100% and 120%) of the nominal sample components concentration (100.0 mg/100 ml of the dexpanthenol, 14.4 mg/100 ml of the phenoxyethanol, 3.2 mg/100 ml of the methylparaben, 0.8 mg/100 ml of the ethylparaben, 0.4 mg/100 ml of the propylparaben, 0.8 mg/100 ml of the butylparaben, 0.4 mg/100 ml of the isobutylparaben).

- ***Selectivity studies solutions.***

For the selectivity studies, the seven standards solutions were prepared by dissolving each standard (i.e. the dexpanthenol, the phenoxyethanol, the methylparaben, the ethylparaben, the propylparaben, the isobutylparaben, the butylparaben) in acetonitrile one by one in seven flasks. Each standard was measured using its wavelength (210 nm or 254 nm).

- ***Linearity studies solutions.***

For the linearity studies, five standard solutions were prepared using *the working standard solution A* at five different concentration levels (80, 90, 100, 110, 120%) of the nominal sample components concentration (100.0 mg/100 ml for the dexpanthenol, 14.4 mg/100 ml for the phenoxyethanol, 3.2 mg/100 ml for the methylparaben, 0.8 mg/100 ml for the ethylparaben, 0.4 mg/100 ml for the propylparaben, 0.8 mg/100 ml for the butylparaben, 0.4 mg/100 ml for the isobutylparaben). Each sample was prepared by diluting in acetonitrile.

## 4.4. Method development

- **Stationary phases**

The chromatographic columns Discovery $C_{18}$ and ZORBAX $C_{18}$ were tested for the separation of the samples.

- **Mobile phases**

During method development, several mobile phases in different ratios were tested:

  - Potassium dihydrogen phosphate buffer and methanol in different ratios 40:60 (v/v), 50:50(v/v), 60:40 (v/v),
  - Potassium dihydrogen phosphate buffer and acetonitrile in different ratios 30:70 (v/v), 50:50 (v/v), 60:40 (v/v), 65:35 (v/v), 67: 33 (v/v), 80:20 (v/v), 85:15 (v/v), 90:10 (v/v).

- **Standard and sample solvent**

Two solvents were tested according to mobile phase development: methanol and acetonitrile.

- **Flow rates**

Three flow rates were tested: 0.30 ml $min^{-1}$, 0.50 ml $min^{-1}$, 1.00 ml $min^{-1}$.

- **Autosampler injection**

Three volumes of autosampler injection 1 µl, 5 µl and 10 µl were tested.

- **Detector**

Diode-Array Detector was tested according to the properties of the sample.

- **Temperature**

Isocratic temperature mode of 25°C was tested.

## 4.5. System Suitability Test

All chromatographic system conditions were tested according to the requirements of The Czech Pharmacopoeia and The European Pharmacopoeia.[34,35]

### 4.5.1. Efficiency

Chromatographic system efficiency was measured using *the working standard solution A*.

**Table 5:** Efficiency.

| Analyzed standard | $t_R$ | $w_h$ | N |
|---|---|---|---|
| **Dexpanthenol** | 1.706 | 0.05 | 6794 |
| **Phenoxyethanol** | 3.648 | 0.08 | 10411 |
| **Methylparaben** | 4.055 | 0.10 | 9843 |
| **Ethylparaben** | 6.234 | 0.14 | 10795 |
| **Propylparaben** | 11.095 | 0.24 | 11508 |
| **Isobutylparaben** | 20.254 | 0.42 | 12022 |
| **Butylparaben** | 21.546 | 0.47 | 11401 |

### 4.5.2. Symmetry factor

Chromatographic system symmetry factor of all peaks was measured using *the working standard solution A*.

**Table 6:** Symmetry factors.

| Analyzed standard | $A_s$ |
|---|---|
| **Dexpanthenol** | 1.292 |
| **Phenoxyethanol** | 1.053 |
| **Methylparaben** | 1.049 |
| **Ethylparaben** | 1.078 |
| **Propylparaben** | 1.089 |
| **Isobutylparaben** | 1.102 |
| **Butylparaben** | 1.075 |

### 4.5.3. Resolution

Resolution was measured using *the working standard solution A*.

**Table 7:** Resolution.

| Analyzed standards | $R_s$ |
|---|---|
| **Phenoxyethanol – Dexpanthenol** | 10.952 |
| **Methylparaben – Phenoxyethanol** | 2.664 |
| **Ethylparaben – Methylparaben** | 10.831 |
| **Propylparaben – Ethylparaben** | 14.910 |
| **Isobutylparaben – Propylparaben** | 15.934 |
| **Butylparaben – Isobutylparaben** | 1.675 |

## 4.6. Method Validation

The method validation is a confirmation process that the method is suited for its intended purpose according to the validation requirements clearly documented by regulatory authorities:

- ICH in *Validation of Analytical Procedures: Test and Methodology Q2(R1)*[1] and
- FDA in *Guidance for Industry: Analytical Procedures and Methods Validation.*[36]

### 4.6.1. Accuracy

*The Accuracy studies solutions* were used for accuracy experiment. The evaluated criteria are based on percent recovery *R (%)*.

Table 8: Results for Accuracy study of the dexpanthenol.

| Conc. level | No. | $c_0$ (mg/100 ml) | $c_i$ (mg/100 ml) | R (%) | SD |
|---|---|---|---|---|---|
| 80 % | 1 | 80.19 | 78.93 | 98.42 | 0.577 |
| | 2 | 78.87 | 78.32 | 99.30 | 0.241 |
| | 3 | 79.26 | 79.03 | 99.75 | 0.137 |
| 100 % | 1 | 98.34 | 98.07 | 99.72 | 0.146 |
| | 2 | 98.05 | 97.73 | 99.67 | 0.219 |
| | 3 | 99.21 | 98.85 | 99.63 | 0.205 |
| 120 % | 1 | 122.97 | 123.54 | 100.46 | 0.233 |
| | 2 | 118.81 | 118.61 | 99.83 | 0.178 |
| | 3 | 117.84 | 117.60 | 99.80 | 0.115 |

Table 9: Results for Accuracy study of the phenoxyethanol.

| Conc. level | No. | $c_0$ (mg/100 ml) | $c_i$ (mg/100 ml) | R (%) | SD |
|---|---|---|---|---|---|
| 80 % | 1 | 11.44 | 11.39 | 99.60 | 0.024 |
| | 2 | 11.30 | 11.32 | 99.95 | 0.015 |
| | 3 | 11.45 | 11.44 | 99.95 | 0.037 |
| 100 % | 1 | 14.42 | 14.43 | 100.10 | 0.010 |
| | 2 | 14.33 | 14.34 | 100.05 | 0.024 |
| | 3 | 14.53 | 14.51 | 99.83 | 0.014 |
| 120 % | 1 | 17.87 | 17.93 | 100.31 | 0.039 |
| | 2 | 17.15 | 17.11 | 99.76 | 0.023 |
| | 3 | 16.93 | 16.94 | 100.04 | 0.006 |

Table 10: Results for Accuracy study of the methylparaben.

| Conc. level | No. | $c_0$ (mg/100 ml) | $c_i$ (mg/100 ml) | R (%) | SD |
|---|---|---|---|---|---|
| 80 % | 1 | 2.4244 | 2.4215 | 99.88 | 0.0074 |
| | 2 | 2.4044 | 2.4041 | 99.99 | 0.0009 |
| | 3 | 2.4208 | 2.4277 | 100.28 | 0.0063 |
| 100 % | 1 | 3.1780 | 3.1742 | 99.88 | 0.0042 |
| | 2 | 3.1540 | 3.1528 | 99.63 | 0.0028 |
| | 3 | 3.1884 | 3.1963 | 100.25 | 0.0040 |
| 120 % | 1 | 3.8456 | 3.8633 | 100.46 | 0.0101 |
| | 2 | 3.7016 | 3.6977 | 99.89 | 0.0040 |
| | 3 | 3.6652 | 3.6633 | 99.95 | 0.0035 |

Table 11: Results for Accuracy study of the ethylparaben.

| Conc. level | No. | $c_0$ (mg/100 ml) | $c_i$ (mg/100 ml) | R (%) | SD |
|---|---|---|---|---|---|
| 80 % | 1 | 0.6128 | 0.6114 | 99.77 | 0.0017 |
| | 2 | 0.6064 | 0.6073 | 100.15 | 0.0005 |
| | 3 | 0.6128 | 0.6129 | 100.02 | 0.0016 |
| 100 % | 1 | 0.7864 | 0.7861 | 99.96 | 0.0012 |
| | 2 | 0.7808 | 0.7809 | 100.01 | 0.0003 |
| | 3 | 0.7928 | 0.7913 | 99.81 | 0.0011 |
| 120 % | 1 | 0.9660 | 0.9666 | 100.06 | 0.0022 |
| | 2 | 0.9252 | 0.9256 | 100.05 | 0.0006 |
| | 3 | 0.9176 | 0.9178 | 100.02 | 0.0002 |

Table 12: Results for Accuracy study of the propylparaben.

| Conc. level | No. | $c_0$ (mg/100 ml) | $c_i$ (mg/100 ml) | R (%) | SD |
|---|---|---|---|---|---|
| 80 % | 1 | 0.3112 | 0.3133 | 100.69 | 0.0021 |
| | 2 | 0.3120 | 0.3120 | 99.99 | 0.0002 |
| | 3 | 0.3164 | 0.3155 | 99.73 | 0.0019 |
| 100 % | 1 | 0.3920 | 0.3919 | 99.97 | 0.0006 |
| | 2 | 0.3904 | 0.3895 | 99.76 | 0.0006 |
| | 3 | 0.3968 | 0.3955 | 99.68 | 0.0007 |
| 120 % | 1 | 0.4912 | 0.4917 | 100.10 | 0.0015 |
| | 2 | 0.4700 | 0.4697 | 99.94 | 0.0002 |
| | 3 | 0.4664 | 0.4661 | 99.94 | 0.0004 |

Table 13: Results for Accuracy study of the isobutylparaben.

| Conc. level | Měř. | $c_0$ (mg/100 ml) | $c_i$ (mg/100 ml) | R (%) | SD |
|---|---|---|---|---|---|
| 80 % | 1 | 0.3132 | 0.3129 | 99.91 | 0.0010 |
| | 2 | 0.3104 | 0.3093 | 99.66 | 0.0009 |
| | 3 | 0.3140 | 0.3148 | 100.24 | 0.0010 |
| 100 % | 1 | 0.3968 | 0.3970 | 100.04 | 0.0014 |
| | 2 | 0.3916 | 0.3941 | 100.64 | 0.0045 |
| | 3 | 0.4012 | 0.3994 | 99.55 | 0.0030 |
| 120 % | 1 | 0.4928 | 0.4935 | 100.14 | 0.0019 |
| | 2 | 0.4692 | 0.4689 | 99.94 | 0.0012 |
| | 3 | 0.4652 | 0.4656 | 100.08 | 0.0015 |

**Table 14**: Results for Accuracy study of the butylparaben.

| Conc. level | No. | $c_0$ (mg/100 ml) | $c_i$ (mg/100 ml) | R (%) | SD |
|---|---|---|---|---|---|
| **80 %** | 1 | 0.6016 | 0.5995 | 99.66 | 0.0011 |
| | 2 | 0.6156 | 0.6141 | 99.75 | 0.0034 |
| | 3 | 0.6012 | 0.6011 | 99.98 | 0.0105 |
| **100 %** | 1 | 0.7752 | 0.7809 | 100.73 | 0.0157 |
| | 2 | 0.7856 | 0.7834 | 99.72 | 0.0170 |
| | 3 | 0.7844 | 0.7840 | 99.95 | 0.0115 |
| **120 %** | 1 | 0.9472 | 0.9482 | 100.11 | 0.0014 |
| | 2 | 0.9098 | 0.9100 | 100.02 | 0.0036 |
| | 3 | 0.9072 | 0.9059 | 99.86 | 0.0030 |

### 4.6.2. Precision

#### *4.6.2.1. Injection Precision (Injection Repeatability)*

For validation of the injection repeatability, *the working standard solution A* was used. The criteria are based on relative standard deviation RSD (%) of the peak area.

**Table 15:** Results for Injection repeatability study.

| No. | D-pantenol | Phenoxy ethanol | M-Paraben | E-Paraben | P-Paraben | IB-Paraben | B-Paraben |
|---|---|---|---|---|---|---|---|
| 1 | 256968975 | 16998372 | 121939505 | 28620153 | 13134429 | 10805090 | 24195897 |
| 2 | 256501680 | 16924320 | 121645390 | 28492283 | 13110453 | 10777517 | 24250538 |
| 3 | 256220244 | 16911036 | 121472832 | 28421879 | 13130267 | 10776494 | 24319481 |
| 4 | 256031570 | 16890654 | 121213332 | 28447098 | 13046019 | 10703113 | 24163862 |
| 5 | 256724094 | 16902620 | 121404091 | 28473865 | 13068099 | 10757827 | 24080357 |
| 6 | 256277089 | 16879582 | 121249046 | 28464917 | 13074154 | 10744543 | 24231254 |
| **Mean** | **256453942** | **16917764** | **121487501** | **28486699** | **13093904** | **10760764** | **24206898** |
| SD | 347795.9 | 42445.4 | 271405.2 | 69642.2 | 36297.0 | 34895.0 | 81435.1 |
| **RSD (%)** | **0.136** | **0.251** | **0.223** | **0.244** | **0.277** | **0.324** | **0.336** |

#### *4.6.2.2. Method Precision (Analysis Repeatability)*

For validation the method precision, *the Panthenol 4% gel sample* was used. The criteria are based on RSD (%).

**Table 16**: Results for Method precision study.

| No. | D-panthenol (%) | Phenoxy ethanol (%) | M-paraben (%) | E-paraben (%) | P-paraben (%) | IB-paraben (%) | B-paraben (%) |
|---|---|---|---|---|---|---|---|
| 1 | 3.99 | 0.564 | 0.1213 | 0.0303 | 0.0150 | 0.0150 | 0.0300 |
| 2 | 4.01 | 0.564 | 0.1216 | 0.0304 | 0.0150 | 0.0151 | 0.0301 |
| 3 | 4.10 | 0.579 | 0.1248 | 0.0311 | 0.0155 | 0.0156 | 0.0308 |
| 4 | 4.02 | 0.567 | 0.1222 | 0.0305 | 0.0151 | 0.0153 | 0.0303 |
| 5 | 4.15 | 0.588 | 0.1266 | 0.0316 | 0.0157 | 0.0158 | 0.0308 |
| 6 | 4.06 | 0.572 | 0.1232 | 0.0308 | 0.0153 | 0.0154 | 0.0302 |
| **Mean** | **4.055** | **0.572** | **0.1233** | **0.0308** | **0.0153** | **0.0154** | **0.0304** |
| SD | 0.0609 | 0.0096 | 0.0021 | 0.0005 | 0.0003 | 0.0003 | 0.0004 |
| **RSD (%)** | **1.502** | **1.671** | **1.672** | **1.610** | **1.883** | **1.959** | **1.153** |

#### *4.6.2.3. Intermediate Precision*

The intermediate precision of the presented chromatographic method was measured by a different analyst, on the same chromatographic system, on a different day, and on the same *the Panthenol 4% gel sample*. The criteria are based on RSD (%) of peak area, mean differences and confidence intervals.

**Table 17**: Results for Intermediate precision study measured by Kateřina Dittrichová (2. 12. 2011).

| No. | D-panthenol (%) | Phenoxy ethanol (%) | M-paraben (%) | E-paraben (%) | P-paraben (%) | IB-paraben (%) | B-paraben (%) |
|---|---|---|---|---|---|---|---|
| 1 | 3.99 | 0.564 | 01213 | 0.0303 | 0.0150 | 0.0150 | 0.0300 |
| 2 | 4.01 | 0.564 | 0.1216 | 0.0304 | 0.0150 | 0.0151 | 0.0301 |
| 3 | 4.10 | 0.579 | 0.1248 | 0.0311 | 0.0155 | 0.0156 | 0.0308 |
| 4 | 4.02 | 0.567 | 0.1222 | 0.0305 | 0.0151 | 0.0153 | 0.0303 |
| 5 | 4.15 | 0.588 | 0.1266 | 0.0316 | 0.0157 | 0.0158 | 0.0308 |
| 6 | 4.06 | 0.572 | 0.1232 | 0.0308 | 0.0153 | 0.0154 | 0.0302 |
| **Mean** | **4.055** | **0.572** | **0.1233** | **0.0308** | **0.0153** | **0.0154** | **0.0304** |
| SD | 0.0609 | 0.0096 | 0.0021 | 0.0005 | 0.0003 | 0.0003 | 0.0004 |
| **RSD (%)** | **1.502** | **1.671** | **1.672** | **1.610** | **1.883** | **1.959** | **1.153** |

**Table 18**: Results for Intermediate precision study measured by Ing. Lubomír Galla (7. 12. 2011).

| No. | D-panthenol (%) | Phenoxy ethanol (%) | M-paraben (%) | E-paraben (%) | P-paraben (%) | IB-paraben (%) | B-paraben (%) |
|---|---|---|---|---|---|---|---|
| 1 | 3.96 | 0.560 | 0.1212 | 0.0303 | 0.0151 | 0.0149 | 0.0295 |
| 2 | 4.03 | 0.569 | 0.1235 | 0.0308 | 0.0153 | 0.0152 | 0.0302 |
| 3 | 3.94 | 0.553 | 0.1203 | 0.0301 | 0.0150 | 0.0149 | 0.0299 |
| 4 | 4.03 | 0.567 | 0.1232 | 0.0308 | 0.0154 | 0.0152 | 0.0302 |
| 5 | 4.06 | 0.571 | 0.1240 | 0.0310 | 0.0155 | 0.0154 | 0.0307 |
| 6 | 4.02 | 0.566 | 0.1230 | 0.0307 | 0.0153 | 0.0153 | 0.0307 |
| **Mean** | **4.007** | **0.564** | **0.1225** | **0.0306** | **0.0153** | **0.0152** | **0.0302** |
| SD | 0.0463 | 0.0067 | 0.0015 | 0.0003 | 0.0002 | 0.0002 | 0.0005 |
| **RSD (%)** | **1.156** | **1.184** | **1.183** | **1.120** | **1.220** | **1.369** | **1.539** |

**Table 19**: List of differences between two measurements performed by K. Dittrichová and Ing. L. Galla.

| Analyzed compound | Mean difference | Precision of difference | Confidence Interval |
|---|---|---|---|
| **Dexpanthenol** | 0.0483 | 0.0674 | -0.0224 – 0.1190 |
| **Phenoxyethanol** | 0.0080 | 0.0115 | -0.0040 – 0.0200 |
| **Methylparaben** | 0.0008 | 0.0024 | -0.0018 – 0.0033 |
| **Ethylparaben** | 0.0002 | 0.0005 | -0.0004 – 0.0007 |
| **Propylparaben** | 0.0000 | 0.0003 | -0.0003 – 0.0003 |
| **Isobutylparaben** | 0.0002 | 0.0003 | -0.0001 – 0.0005 |
| **Butylparaben** | 0.0002 | 0.0005 | -0.0003 – 0.0007 |

### 4.6.3. Specificity and Selectivity

To confirm specificity and selectivity of the chromatographic method, the seven *Selectivity studies solutions*, *the working standard solution A*, *the Panthenol 4% gel sample*, *the Placebo Panthenol 4% gel without dexpanthenol sample*, *the Placebo Panthenol 4% gel without dexpanthenol and preservatives sample* were used.

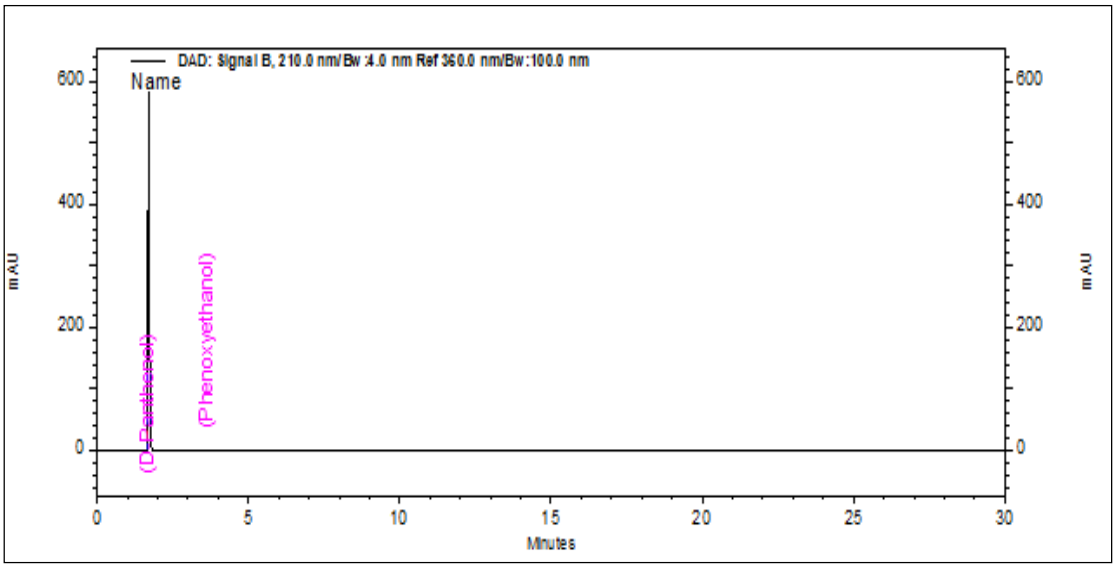


**Figure 4**: Chromatogram of the dexpanthenol standard, 210 nm.

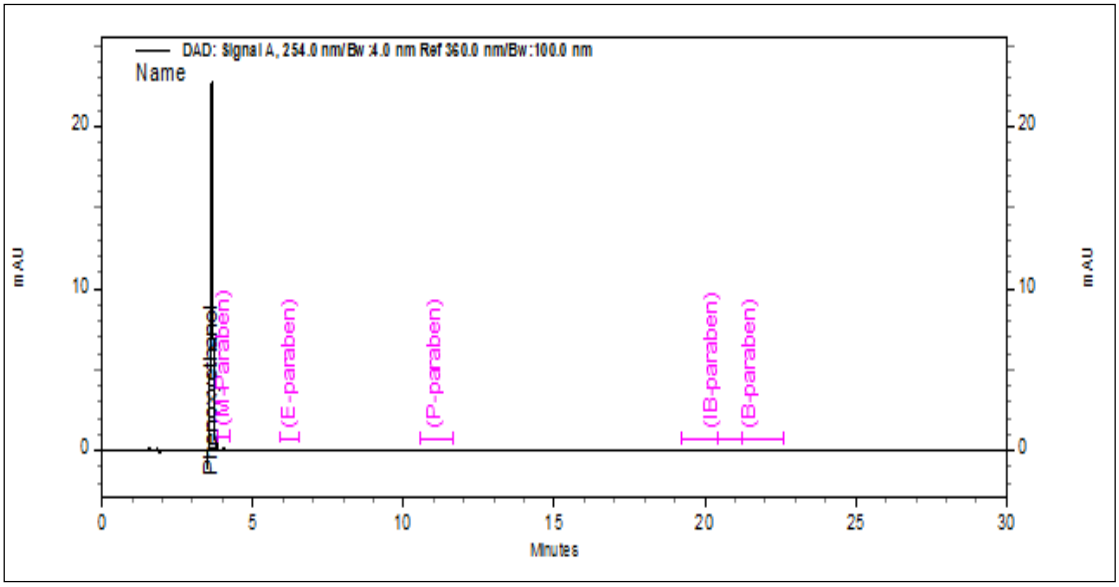


**Figure 5**: Chromatogram of the phenoxyethanol standard, 254 nm.

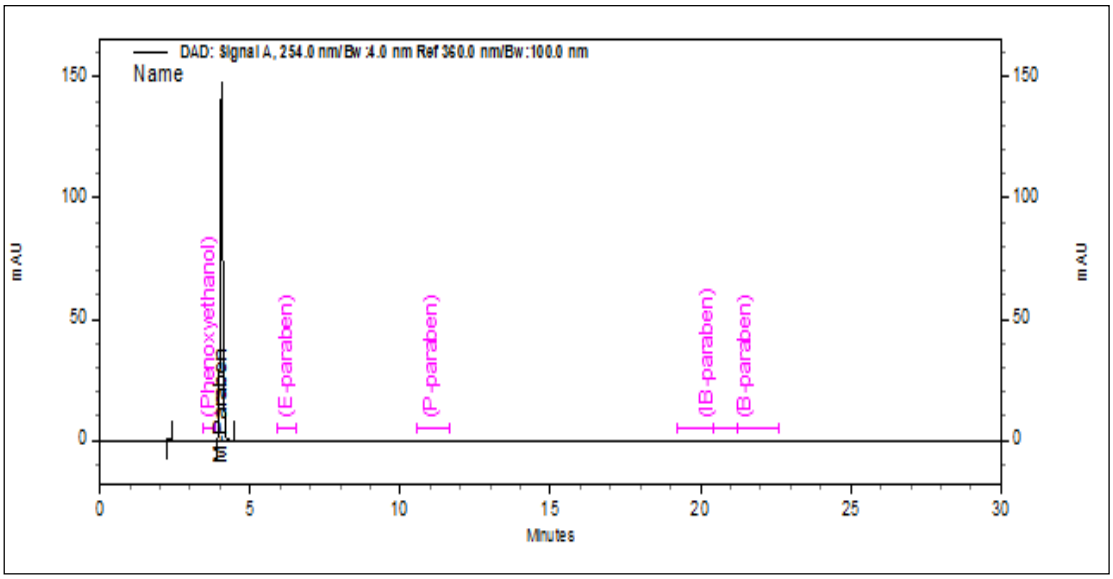


**Figure 6**: Chromatogram of the methylparaben standard, 254 nm.

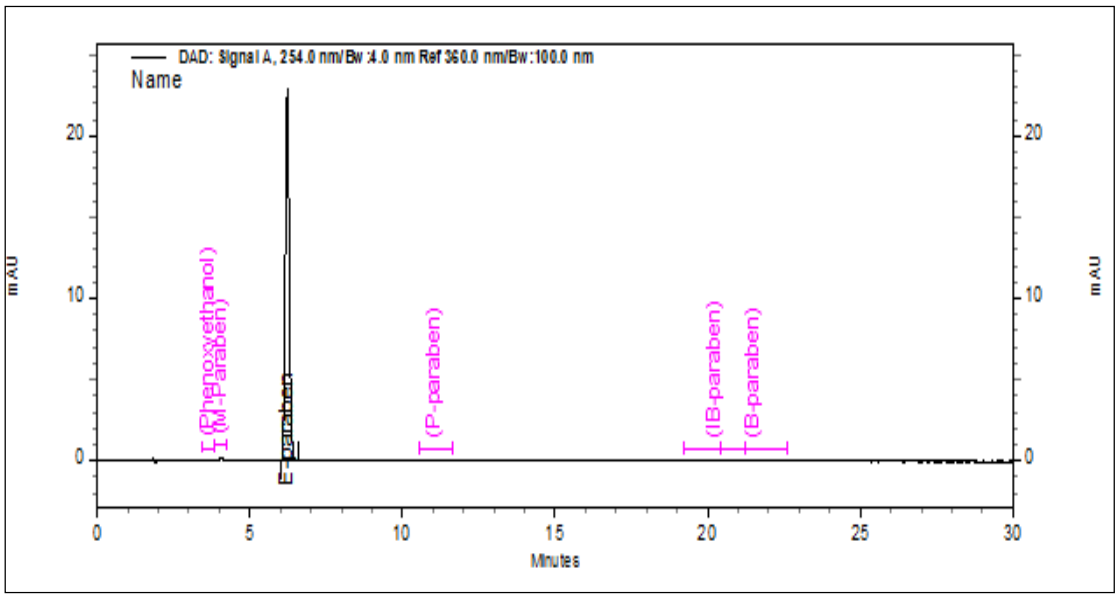


**Figure 7**: Chromatogram of the ethylparaben standard, 254 nm.

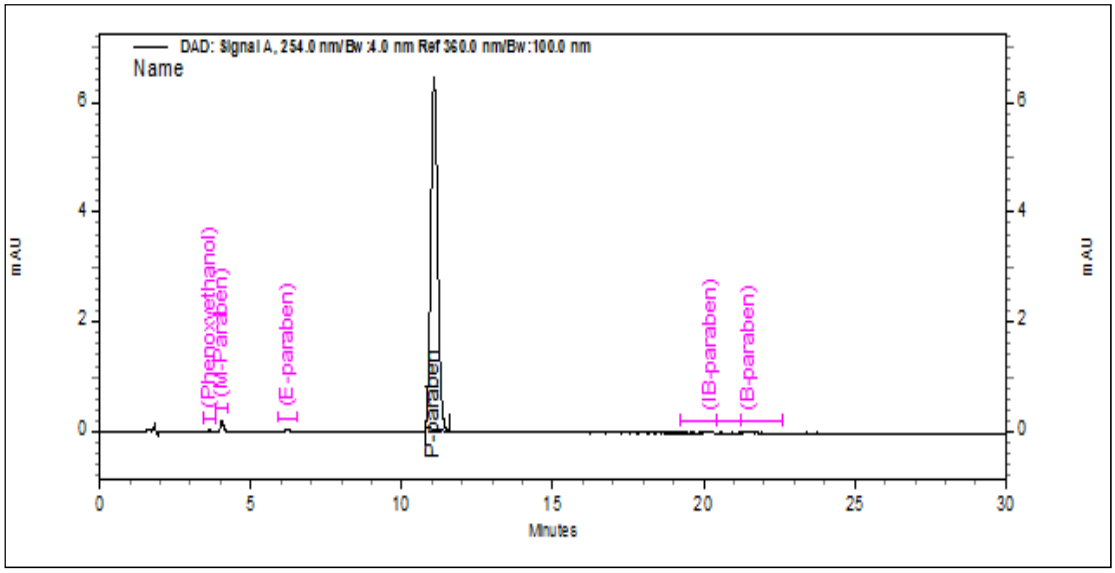


**Figure 8**: Chromatogram of the propylparaben standard, 254 nm.

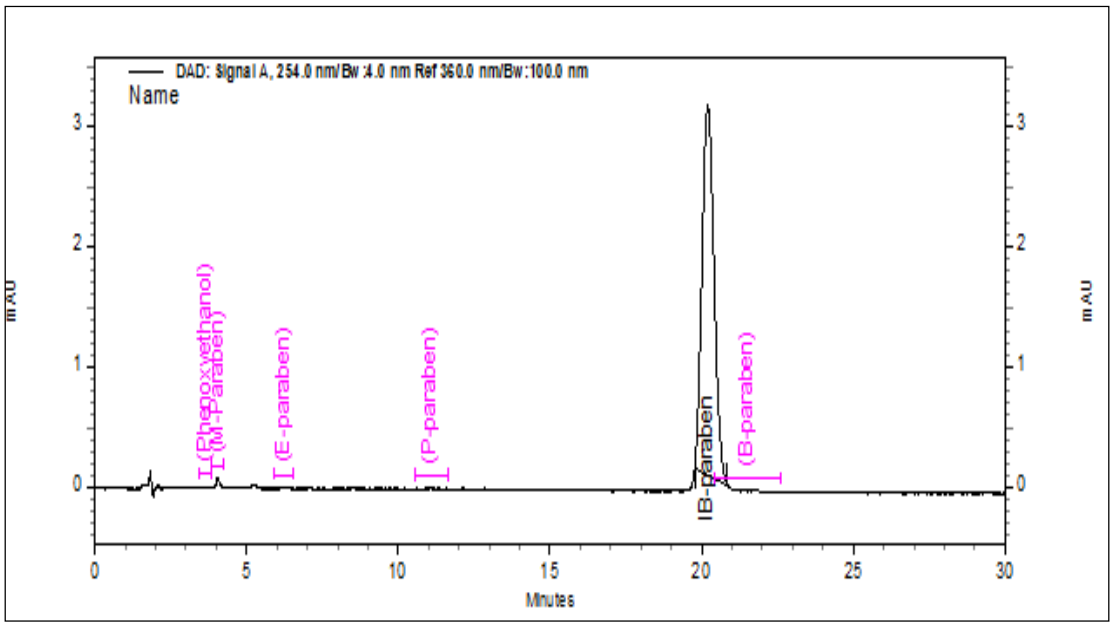


**Figure 9**: Chromatogram of the isobutylparaben standard, 254 nm.

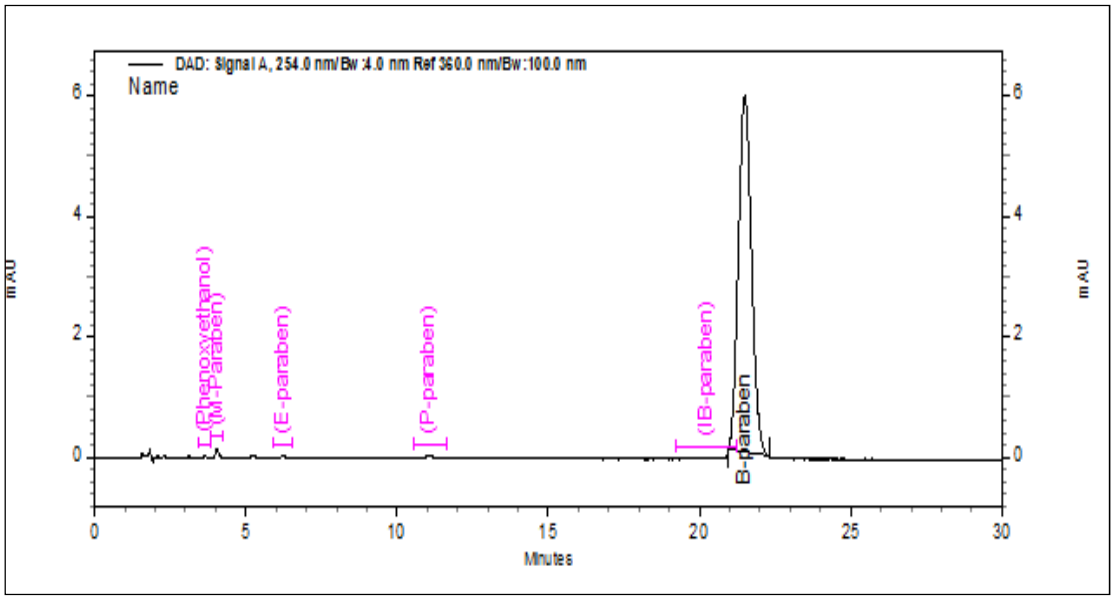


**Figure 10**: Chromatogram of the butylparaben standard, 254 nm.

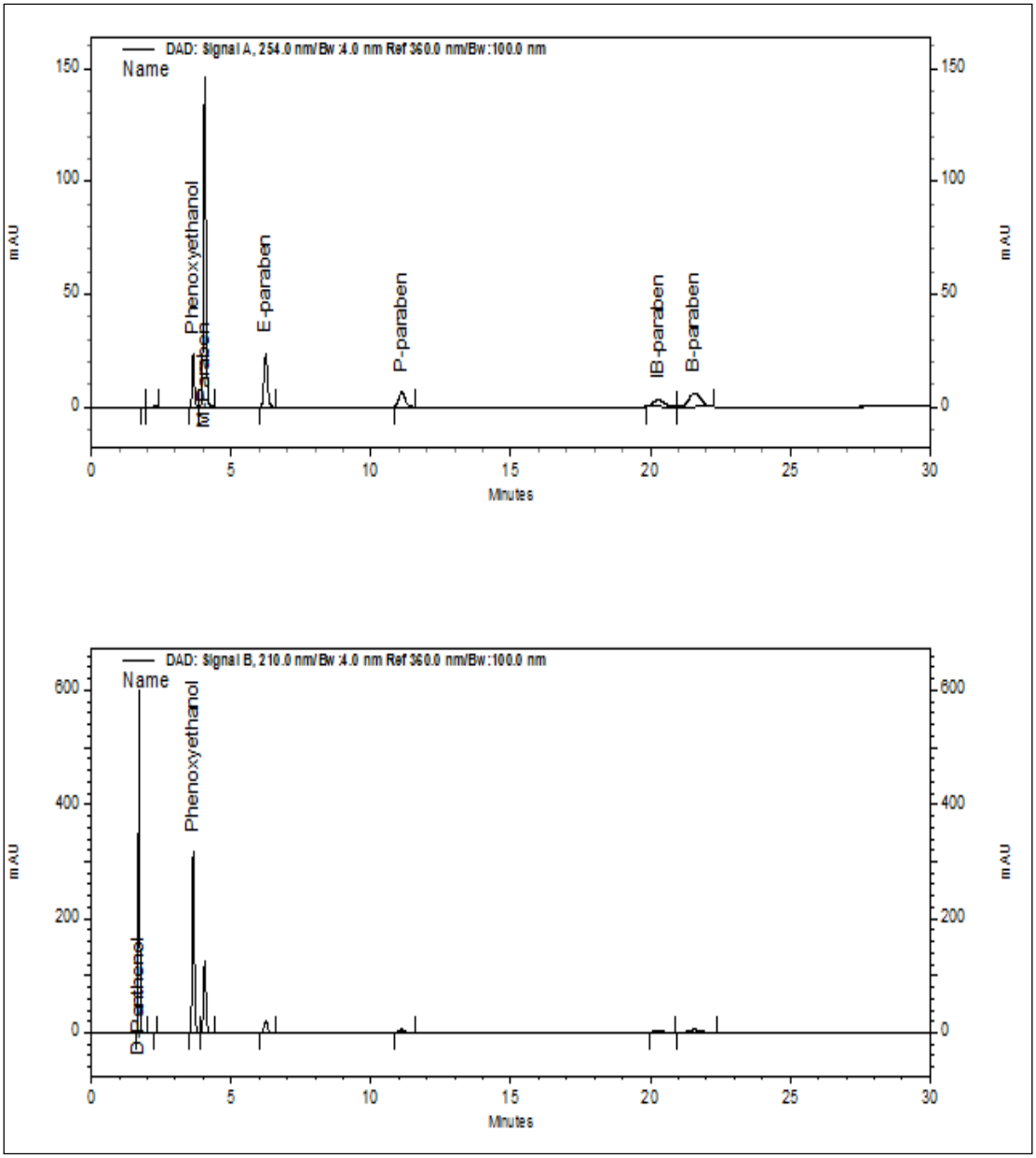


**Figure 11**: Chromatograms of *the working standard solution A*, 254 nm and 210 nm.

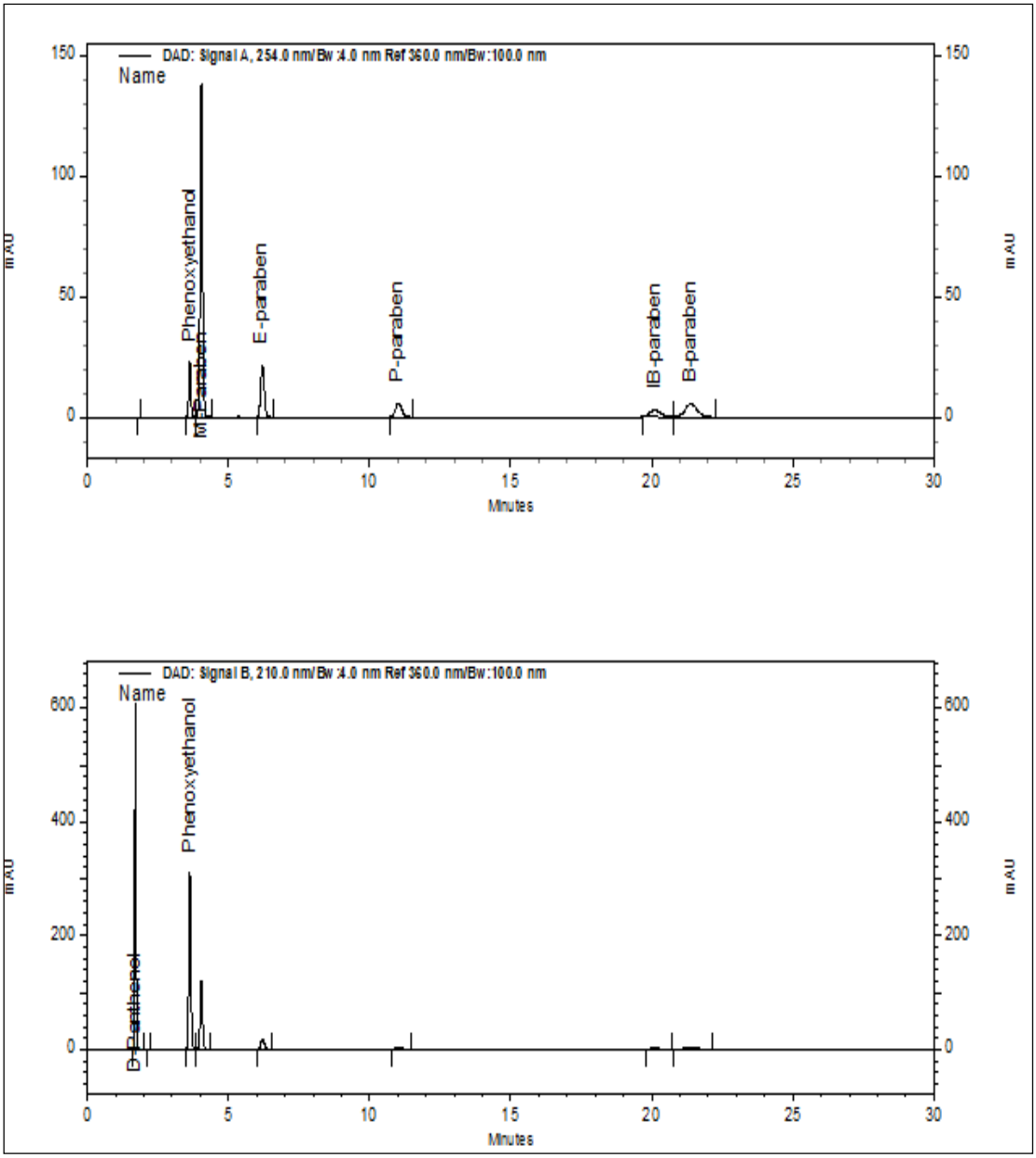


**Figure 12**: Chromatograms of *The Panthenol 4% gel sample*, 254 nm and 210 nm.

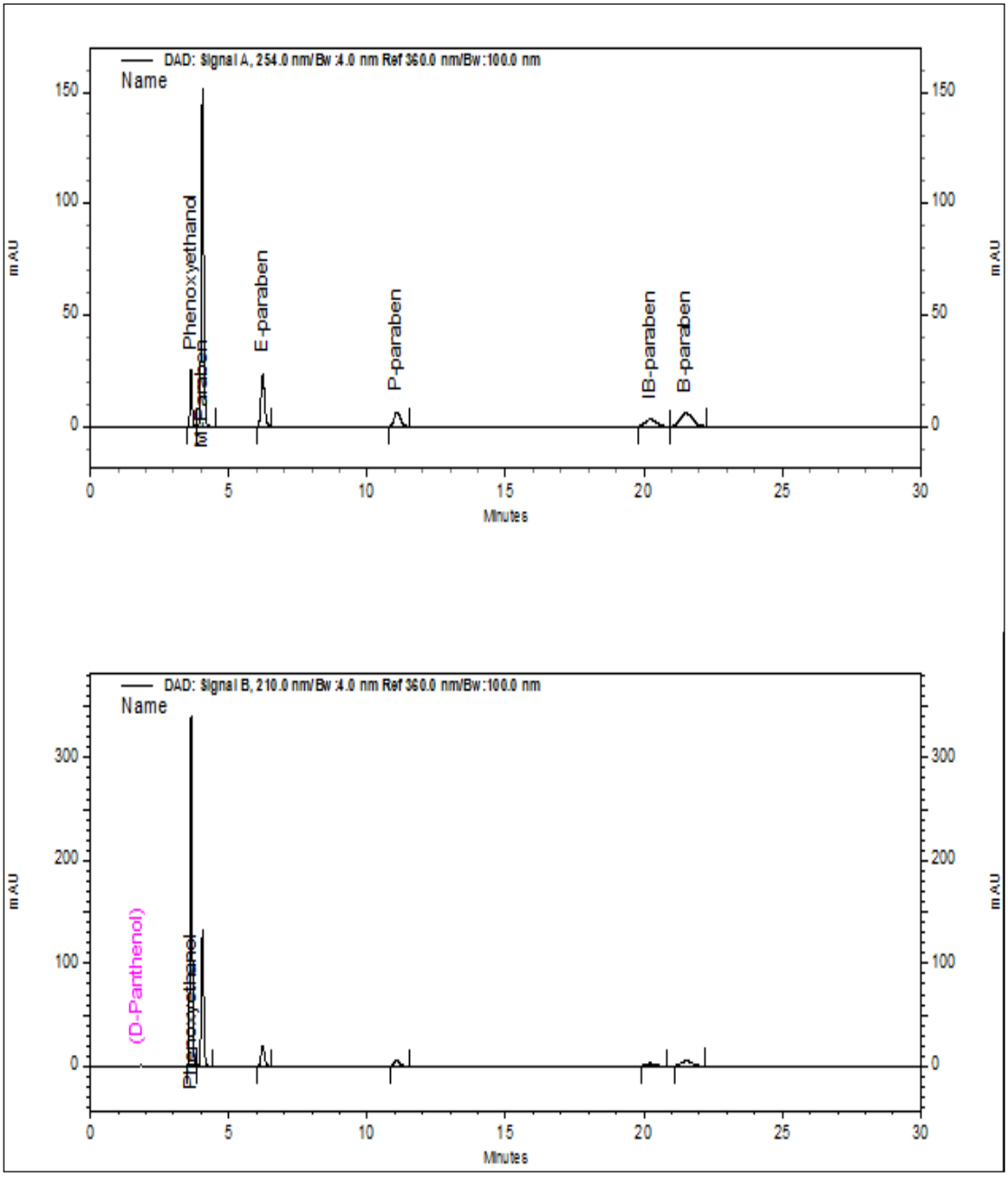


**Figure 13**: Chromatograms of *the Placebo Panthenol 4% gel without dexpanthenol sample*, 254 nm and 210 nm.

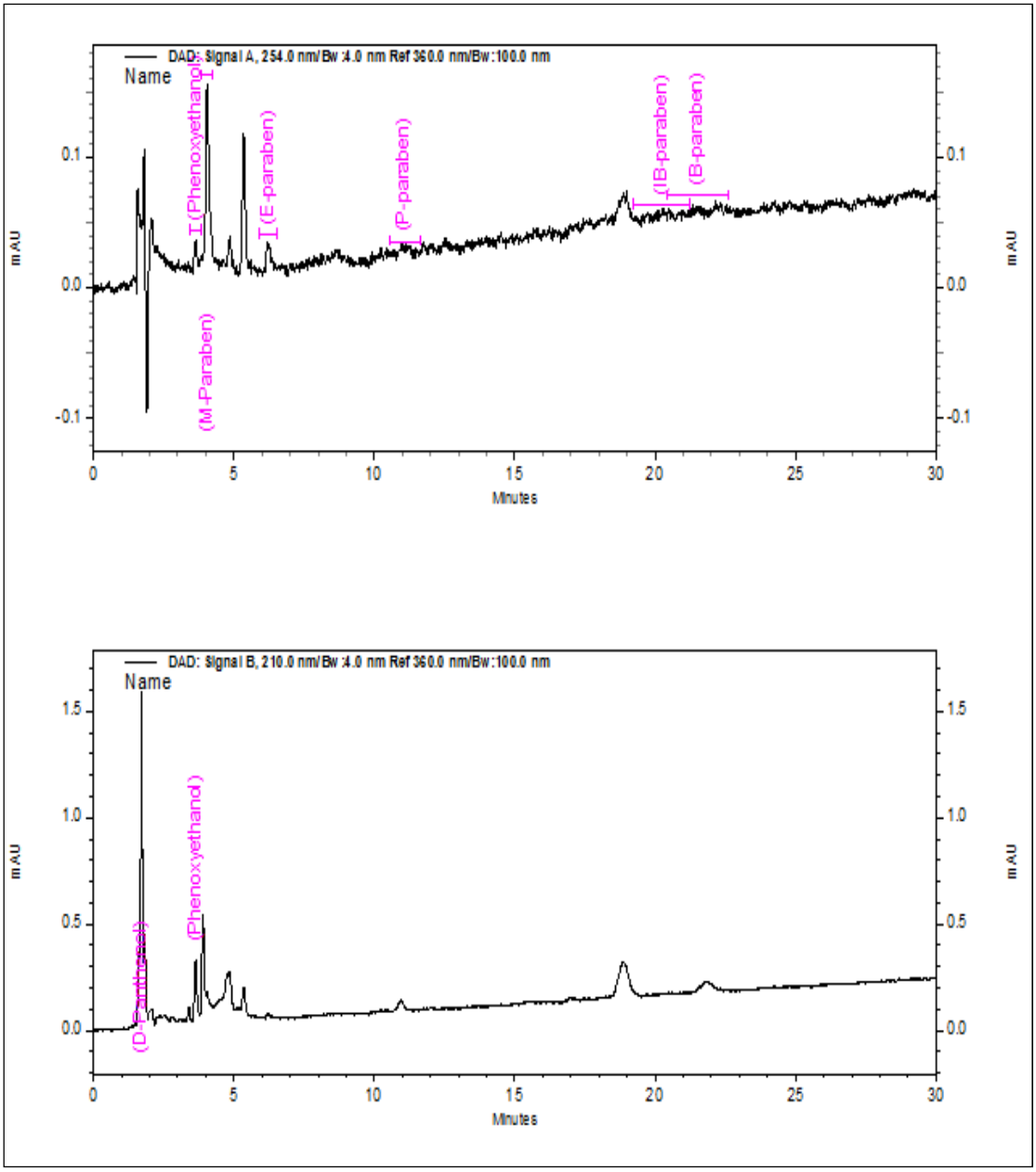


**Figure 14**: Chromatograms of *the Placebo Panthenol 4% gel without dexpanthenol and preservatives sample,* 254 nm and 210 nm.

### 4.6.4. Linearity

*Linearity studies solutions* were used for performing method Linearity experiment. The criteria are based on the value of correlation coefficient *R* which should be at least 0.9900.

#### *4.6.4.1. Dexpanthenol*

**Table 20**: Table of peak areas of the dexpanthenol.

| Conc. level | Concentration (mg/100 ml) | Peak area | | | Mean peak area |
|---|---|---|---|---|---|
| | | No. 1 | No. 2 | No. 3 | |
| **80 %** | 80.54 | 212838905 | 212649811 | 212772454 | 212753723 |
| **90 %** | 90.14 | 236795614 | 236852998 | 236869085 | 236839232 |
| **100 %** | 99.52 | 256520553 | 256501535 | 256542107 | 256521398 |
| **110 %** | 110.24 | 283665763 | 283506853 | 283596247 | 283589621 |
| **120 %** | 120.76 | 309718279 | 309387155 | 310144523 | 309749986 |


**Figure 15**: Calibration curve of the dexpanthenol.

**Table 21:** Table of calibration curve parameters of the dexpanthenol.

| **Regression line** | **y = 2395940.2x + 19721741.8** |
|---|---|
| **Correlation coefficient** | **R = 0.99961** |
| y-intercept of the regression line | 19721741.82483 |
| The slope of the regression line | 2395940.24649 |
| Confidence interval of the y-intercept | 7214486.46958 – 32228997.18008 |
| Confidence interval of the slope | 2272404.76609 – 2519475.7269 |

#### *4.6.4.2. Phenoxyethanol*

**Table 22**: Table of peak areas of the phenoxyethanol.

| Conc. level | Concentration (mg/100 ml) | Peak area | | | Mean peak area |
|---|---|---|---|---|---|
| | | No. 1 | No. 2 | No. 3 | |
| **80 %** | 11.41 | 13631325 | 13612138 | 13635891 | 13626451 |
| **90 %** | 13.21 | 15743949 | 15752383 | 15754595 | 15750309 |
| **100 %** | 14.23 | 16931217 | 16943803 | 16941086 | 16938702 |
| **110 %** | 16.02 | 19053508 | 19073909 | 19069946 | 19065788 |
| **120 %** | 17.19 | 20508453 | 20448951 | 20521660 | 20499688 |

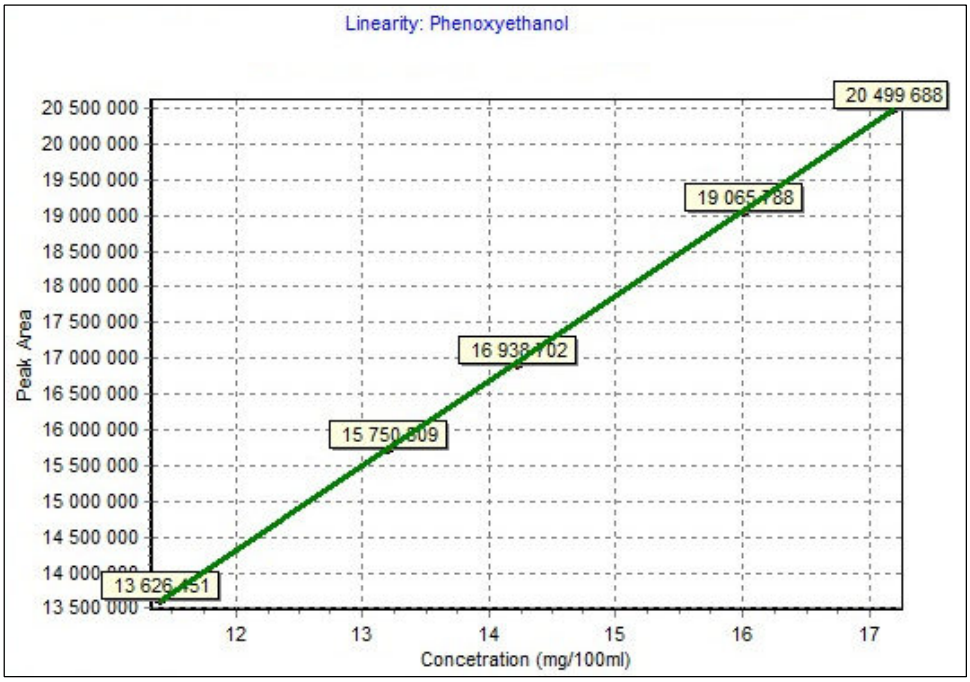


**Figure 16**: Calibration curve of the phenoxyethanol.

**Table 23:** Table of calibration curve parameters of the phenoxyethanol.

| **Regression line** | **y = 1187165.9x + 66752.9** |
|---|---|
| **Correlation coefficient** | **R = 0.99997** |
| y-intercept of the regression line | 66752.8928 |
| The slope of the regression line | 1187165.8831 |
| Confidence interval of the y-intercept | (-174165.8676) – 307671.65321 |
| Confidence interval of the slope | 1170614.21732 – 1203717.54888 |

#### *4.6.4.3. Methylparaben*

**Table 24**: Table of peak areas of the methylparaben.

| Conc. level | Concentration (mg/100 ml) | Peak area | | | Mean peak area |
|---|---|---|---|---|---|
| | | No. 1 | No. 2 | No. 3 | |
| **80 %** | 2.5728 | 98829375 | 98814357 | 98824057 | 98822596 |
| **90 %** | 2.8384 | 107957115 | 107928520 | 108254053 | 108046563 |
| **100 %** | 3.1940 | 121302899 | 121434582 | 121478302 | 121405261 |
| **110 %** | 3.4952 | 130398132 | 130427685 | 130438866 | 130421561 |
| **120 %** | 3.8512 | 145078205 | 144946564 | 145072251 | 145032340 |

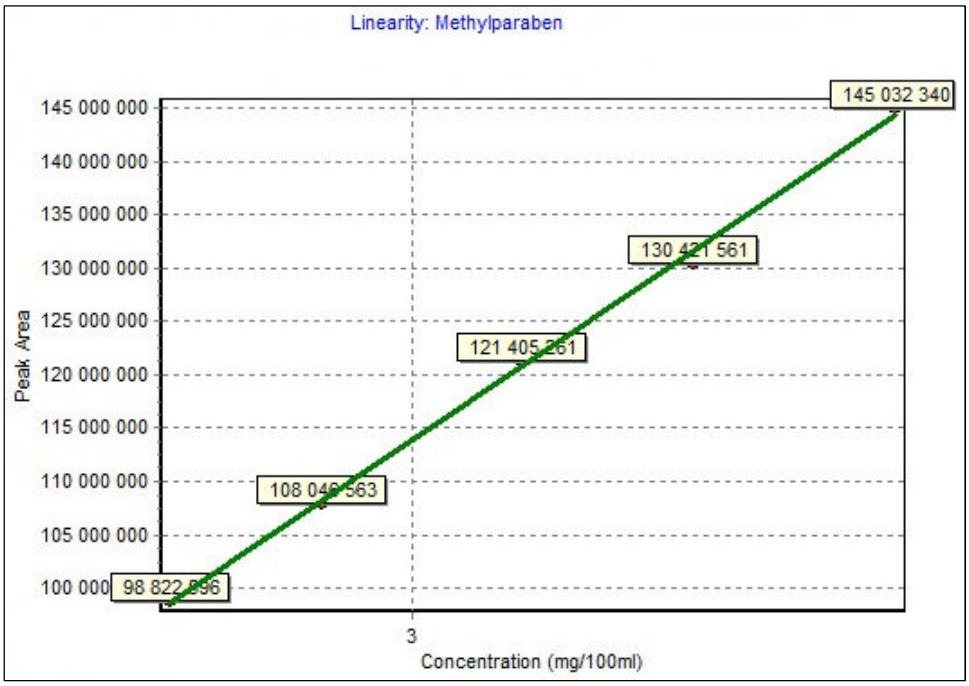


**Figure 17**: Calibration curve of the methylparaben.

**Table 25:** Table of calibration curve parameters of the methylparaben.

| **Regression line** | **y = 35761683.2x + 6654450.9** |
|---|---|
| **Correlation coefficient** | **R = 0.99915** |
| y-intercept of the regression line | 6654450.92431 |
| The slope of the regression line | 35761683.24046 |
| Confidence interval of the y-intercept | (-2073199.79098) – 15382101.6396 |
| Confidence interval of the slope | 33053415.88177 – 38469950.59914 |

#### *4.6.4.4. Ethylparaben*

**Table 26**: Table of peak areas of the ethylparaben.

| Conc. level | Concentration (mg/100 ml) | Peak area | | | Mean peak area |
|---|---|---|---|---|---|
| | | No. 1 | No. 2 | No. 3 | |
| **80 %** | 0.6548 | 23226730 | 23199612 | 23224552 | 23216965 |
| **90 %** | 0.7092 | 24914623 | 24899522 | 24953789 | 24922645 |
| **100 %** | 0.8104 | 28470739 | 28489718 | 28434813 | 28465090 |
| **110 %** | 0.8564 | 29998232 | 30028340 | 30019000 | 30015191 |
| **120 %** | 0.9560 | 33273661 | 33298503 | 33288775 | 33286980 |

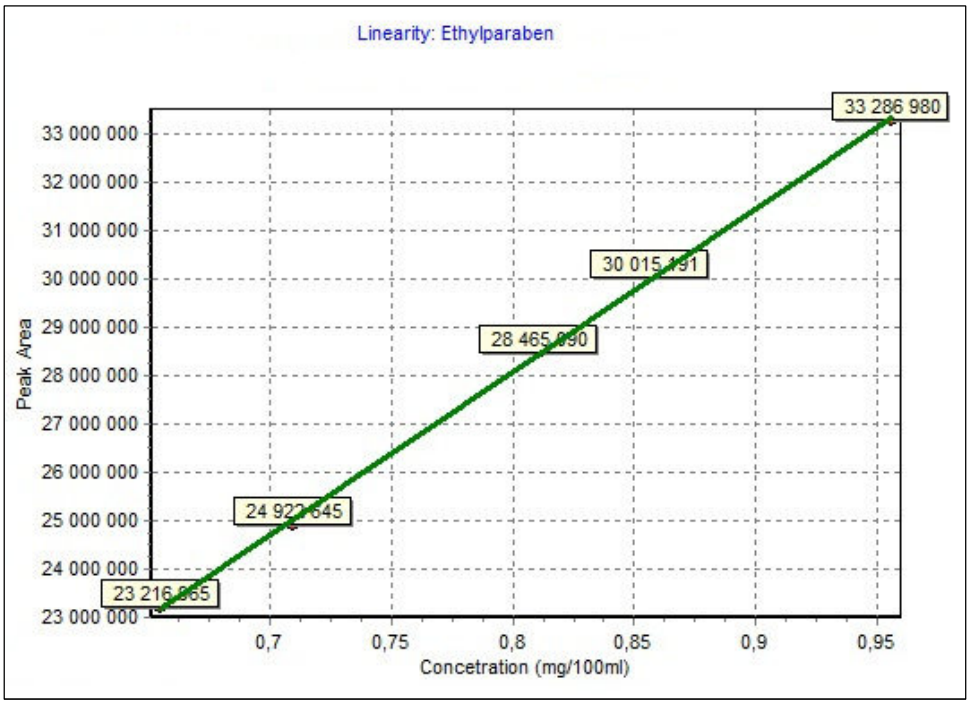


**Figure 18**: Calibration curve of the ethylparaben.

**Table 27:** Table of calibration curve parameters of the ethylparaben.

| **Regression line** | **y = 33678956.8x + 1127120.9** |
|---|---|
| **Correlation coefficient** | **R = 0.99989** |
| y-intercept of the regression line | 1127120.91639 |
| The slope of the regression line | 33678956.82871 |
| Confidence interval of the y-intercept | 373053.14116 – 1881188.69162 |
| Confidence interval of the slope | 32741605.61189 – 34616308.04553 |

#### *4.6.4.5. Propylparaben*

**Table 28**: Table of peak areas of the propylparaben.

| Conc. level | Concentration (mg/100 ml) | Peak area | | | Mean peak area |
|---|---|---|---|---|---|
| | | No. 1 | No. 2 | No. 3 | |
| **80 %** | 0.3144 | 10168522 | 10090303 | 10171673 | 10143499 |
| **90 %** | 0.3544 | 11458045 | 11504934 | 11488432 | 11483804 |
| **100 %** | 0.4076 | 13071399 | 13097915 | 13063480 | 13077598 |
| **110 %** | 0.4284 | 13627507 | 13625050 | 13609303 | 13620620 |
| **120 %** | 0.4900 | 15532146 | 15493706 | 15515622 | 15513825 |

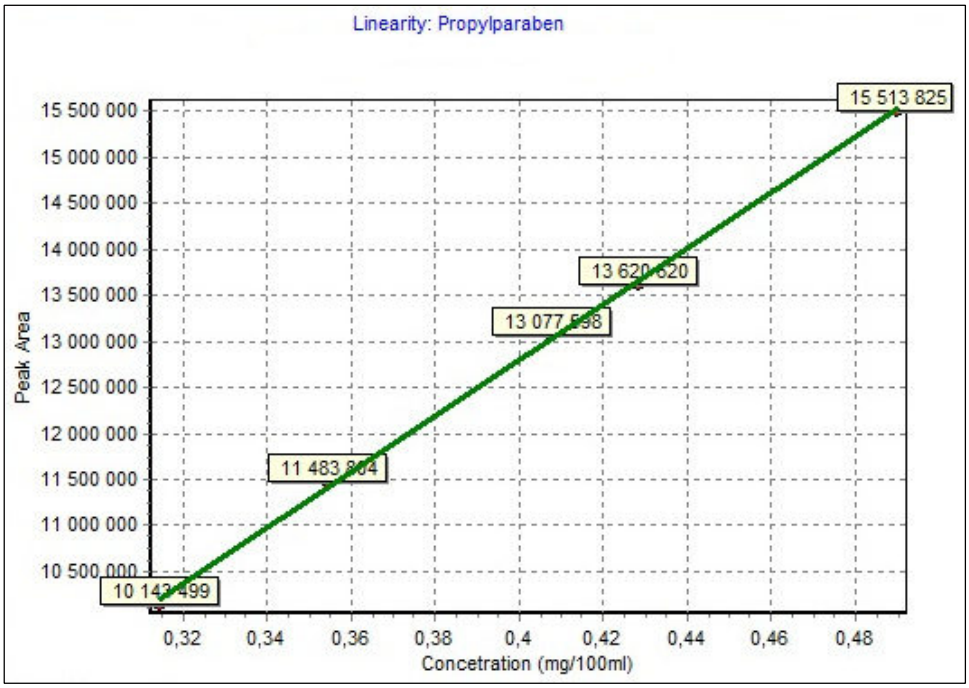


**Figure 19**: Calibration curve of the propylparaben.

**Table 29:** Table of calibration curve parameters of the propylparaben.

| **Regression line** | **y = 30323350.8x + 670065.1** |
|---|---|
| **Correlation coefficient** | **R = 0.99964** |
| y-intercept of the regression line | 670065.09034 |
| The slope of the regression line | 30323350.81962 |
| Confidence interval of the y-intercept | 65164.80369 – 1274965.37699 |
| Confidence interval of the slope | 28824355.42312 – 31822346.21611 |

#### *4.6.4.6. Isobutylparaben*

**Table 30**: Table of peak areas of the isobutylparaben.

| Conc. level | Concentration (mg/100 ml) | Peak area | | | Mean peak area |
|---|---|---|---|---|---|
| | | No. 1 | No. 2 | No. 3 | |
| **80 %** | 0.3276 | 9134064 | 9164393 | 9250894 | 9183117 |
| **90 %** | 0.3656 | 10230582 | 10147903 | 10168337 | 10182241 |
| **100 %** | 0.3904 | 10729123 | 10711552 | 10722450 | 10721042 |
| **110 %** | 0.4312 | 11817905 | 11791791 | 11786631 | 11798776 |
| **120 %** | 0.4748 | 12957660 | 13018682 | 13038546 | 13004963 |

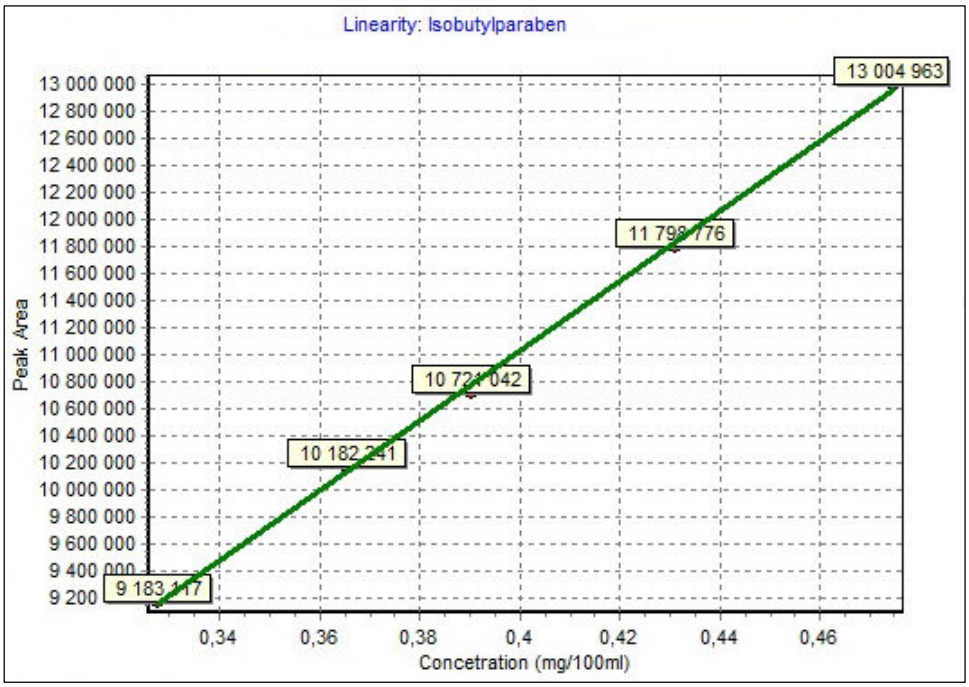


**Figure 20**: Calibration curve of the isobutylparaben.

**Table 31:** Table of calibration curve parameters of the isobutylparaben.

| **Regression line** | **y = 25810705.6x + 708464.0** |
|---|---|
| **Correlation coefficient** | **R = 0.99950** |
| y-intercept of the regression line | 708463.9985 |
| The slope of the regression line | 25810705.57663 |
| Confidence interval of the y-intercept | 105430.619 – 1311497.37801 |
| Confidence interval of the slope | 24307431.58741 – 27313979.56585 |

#### *4.6.4.7. Butylparaben*

**Table 32**: Table of peak areas of the butylparaben.

| Conc. level | Concentration (mg/100 ml) | Peak area | | | Mean peak area |
|---|---|---|---|---|---|
| | | No. 1 | No. 2 | No. 3 | |
| **80 %** | 0.6380 | 18514791 | 18390349 | 18440715 | 18448618 |
| **90 %** | 0.7244 | 20952212 | 20990676 | 21281699 | 21074862 |
| **100 %** | 0.8136 | 24433228 | 24312671 | 24178375 | 24308091 |
| **110 %** | 0.8648 | 25709350 | 25688651 | 25760800 | 25719267 |
| **120 %** | 0.9440 | 28150857 | 28029980 | 28154151 | 28111663 |

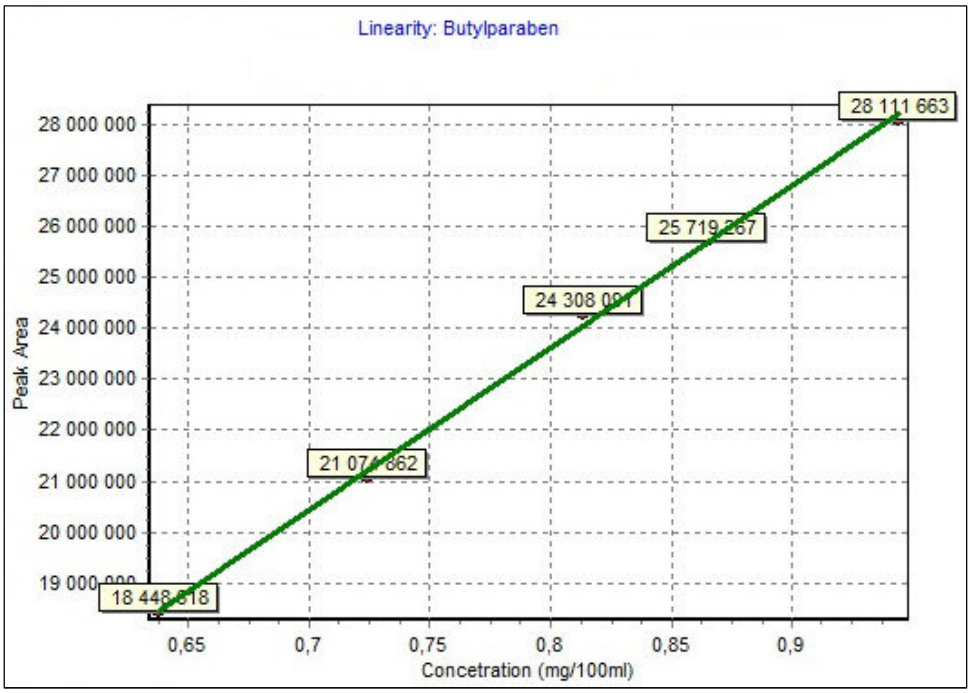


**Figure 21**: Calibration curve of the butylparaben.

**Table 33**: Table of calibration curve parameters of the butylparaben.

| **Regression line** | **y = 31929829.6x + (-1914230.0)** |
|---|---|
| **Correlation coefficient** | **R = 0.99920** |
| y-intercept of the regression line | (-1914229.98156) |
| The slope of the regression line | 31929829.57935 |
| Confidence interval of the y-intercept | (-3804803.026595) – (-23656.69717) |
| Confidence interval of the slope | 29578617.36139 – 34281041.79732 |

# Chapter 5 Results and discussion

## 5.1. Chromatographic conditions for sample analysis

The HPLC system consisting of the Agilent Infinity 1290 liquid chromatograph was operated at an isocratic temperature mode of 25°C. The spectrophotometric detector Diode-Array detector (Agilent Technologies) was operated at two wavelengths (210 nm for the dexpanthenol and 254 nm for the phenoxyethanol and the preservatives). Column Discovery $C_{18}$ (Sigma Aldrich) was selected as the most suitable column for separation process of the dexpanthenol, the phenoxyethanol, and the preservatives due to the physical and chemical properties of the samples, as well as the price and their accessibility.

The decision to utilize potassium dihydrogen phosphate buffer as one component of the mobile phase was made according to the article written by Klejdus, Petrlová, Potěšil et al. (2003). They states that the mobile phases containing potassium dihydrogen phosphate or sodium acetate as buffering compounds are obviously recommended as the most suitable to assure the optimal chromatographic separation of water-soluble vitamins.[42]

The composition of the mobile phase was tested according to the physical and chemical properties of the samples. The final mobile phase containing potassium dihydrogen phosphate buffer and acetonitrile (67:33, v/v) was selected as the most effective composition of the mobile phase because peaks of all analyzed compounds showed sufficient System Suitability parameters, such as efficiency, symmetry, resolution, and acceptable retention times (see paragraphs 5.2. – 5.4.). The flow of the mobile phase was 1.00 ml $min^{-1}$ and an injection volume of 5 µl was used. Acetonitrile was chosen as the most suitable solvent according to the selected mobile phase.

The separation meets the requirements of the Czech Pharmacopoeia[34], as well as the European Pharmacopoeia[35], in that these conditions were selected as most suitable for this experiment. Peaks of all analyzed compounds were

symmetric, well-separated on a baseline and analysis was relatively fast enough for sufficient recovery.

## 5.2. System Suitability Test

Three basic system suitability parameters were evaluated: efficiency, symmetry factor, and resolution (see Tables 5 – 7). The efficiency was calculated by the equation written in paragraph 3.4.1. The numbers of the theoretical plates ($N$) of all compounds were greater than 6000 units. The symmetry factor was calculated by the equation written in paragraph 3.4.2. and should be in interval from 0.8 to 1.5 for highly symmetric peaks. The determined symmetry factors of all compounds were in the interval from 1.1 to 1.3. The resolution of peaks was calculated by the equation written in paragraph 3.4.3. The determined values of resolution of peaks were greater than 1.5. The system suitability test shows that all chromatographic conditions were met according to the requirements of both the Czech Pharmacopoeia[34] and the European Pharmacopoeia[35].

## 5.3. Method Validation

To confirm accuracy of the chromatographic method, the recovery study was carried out by spiking three concentration level samples three times for each analyzed compound. Tables 8 – 14 summarize the accuracy results, expressed as percent recovery (calculated by the equation written in paragraph 3.5.1) and standard deviation. All percent recoveries are within the values of 98.00 % and 101.00 %. The method shows good recovery.

The precision of the chromatographic method was performed as injection precision, method precision and intermediate precision. The results obtained from these analyses are listed in Tables 15 – 19. The percent relative standard deviation is not greater than 0.40 % for six injections of all analyzed compounds in the injection precision experiment and is not greater than 2.00 % for six injections of all analyzed compounds in the method precision experiment. The mean differences between two measurements obtained by different chemists are 0.0483 for the dexpanthenol, 0.0080 for the phenoxyethanol and less than 0.0008 for each preservative. The confidence interval includes zero value. The

intermediate precision of this chromatographic method performs statistically equivalent results.

The specificity and the selectivity of the method, the standards, the samples and the placebos were tested. From the chromatograms (Figures 2 – 12), it is evident there was good separation of all compounds. No visible interference in the elution zone of the analyzed compounds occurred.

For confirmation method linearity, each concentration level (80, 90, 100, 110, 120 % of a nominal sample components concentration) of each analyzed compound was injected three times (see Tables 20, 22, 24, 26, 28, 30 and 32). The calibration curves, the regression lines, the correlation coefficients, the y-intercepts, the slopes of the regression lines and the confidence intervals were obtained (see Tables 21, 23, 25, 27, 29, 31 and 33). The correlation coefficient of each compound is at least 0.99900. Calibration curves are linear in stated concentration levels; the chromatographic system is able to obtain a response (peak area) that is proportional to the concentration (amount) of the analyzed compound in the sample.

The chromatographic method has been sufficiently validated.

## 5.4. Identification

The maximum of absorption (from UV spectrum) was necessary to measure in order to precise identification the wavelengths where the compounds have the highest possible absorption. Selected maxima of absorption show the wavelength that is the best compromise between the highest possible absorption and number of analyzed compounds. According to the figures of maximal absorption (see Figures 22 – 28), the wavelength of 210 nm is selected as the most suitable for determination of the dexpanthenol and the wavelength of 254 nm is selected as the most suitable for determination of the phenoxyethanol and all preservatives.

For more accurate and specific qualitative evaluation it was necessary to measure and compare UV spectra of all analyzed compounds in a standard solution and a sample solution (*the working standard solution A* and *the Panthenol 4% gel sample).* It was required to use the same chromatographic system conditions for both measurements.

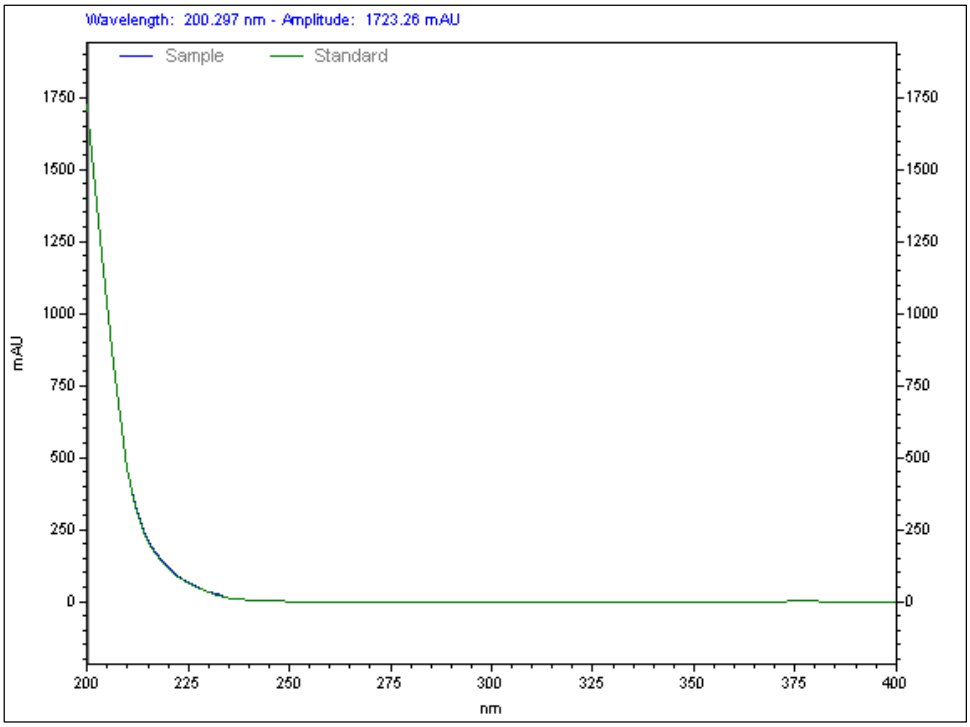


**Figure 22:** UV spectrum of the dexpanthenol.

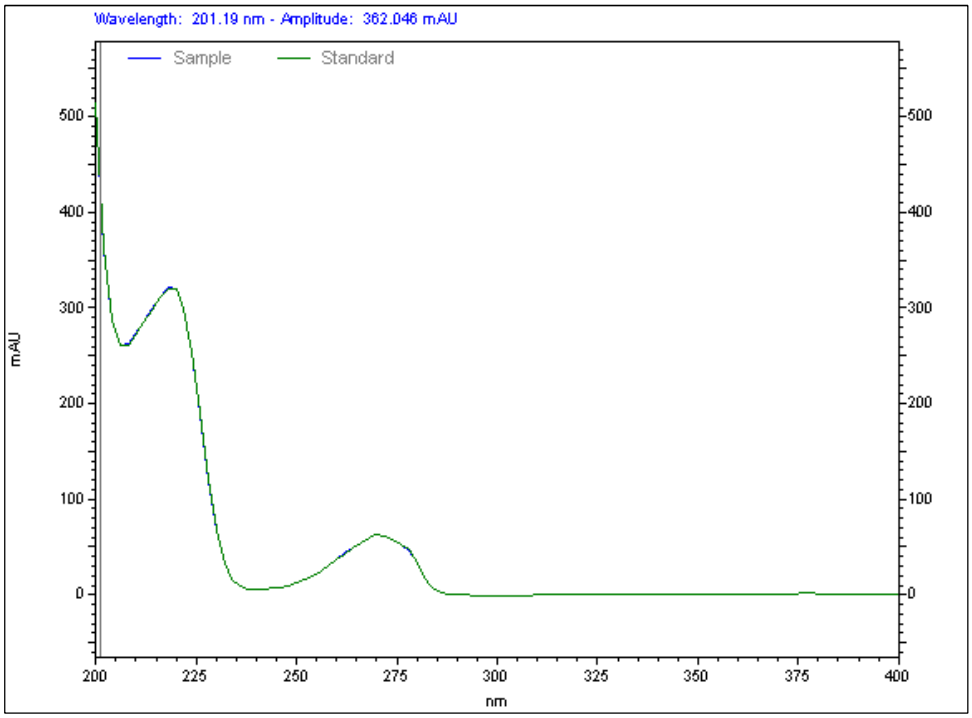


**Figure 23:** UV spectrum of the phenoxyethanol.

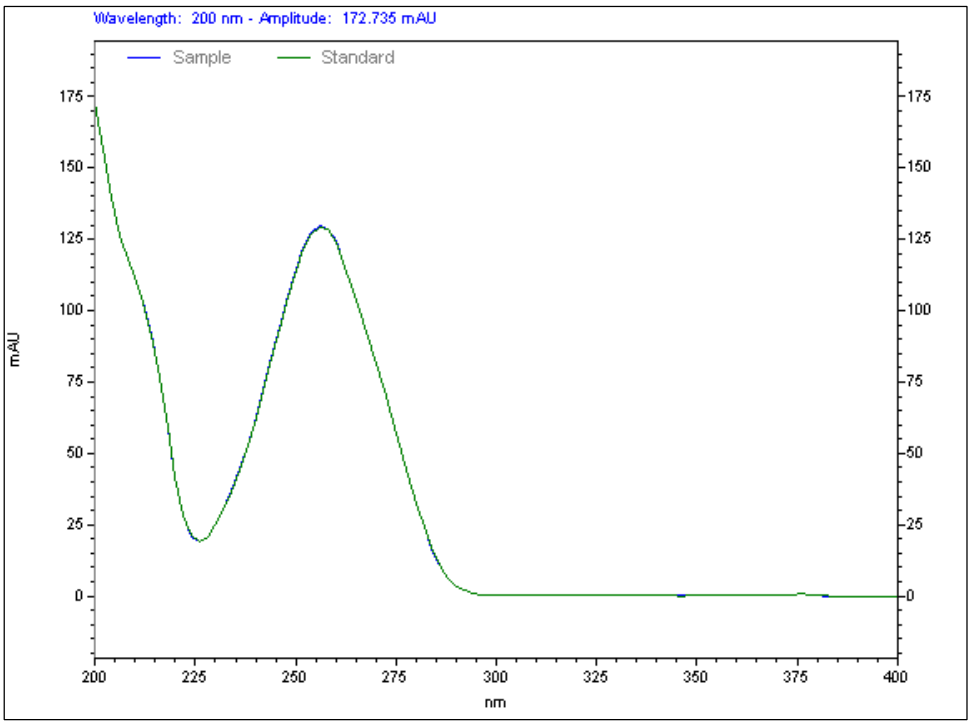


**Figure 24:** UV spectrum of the methylparaben.

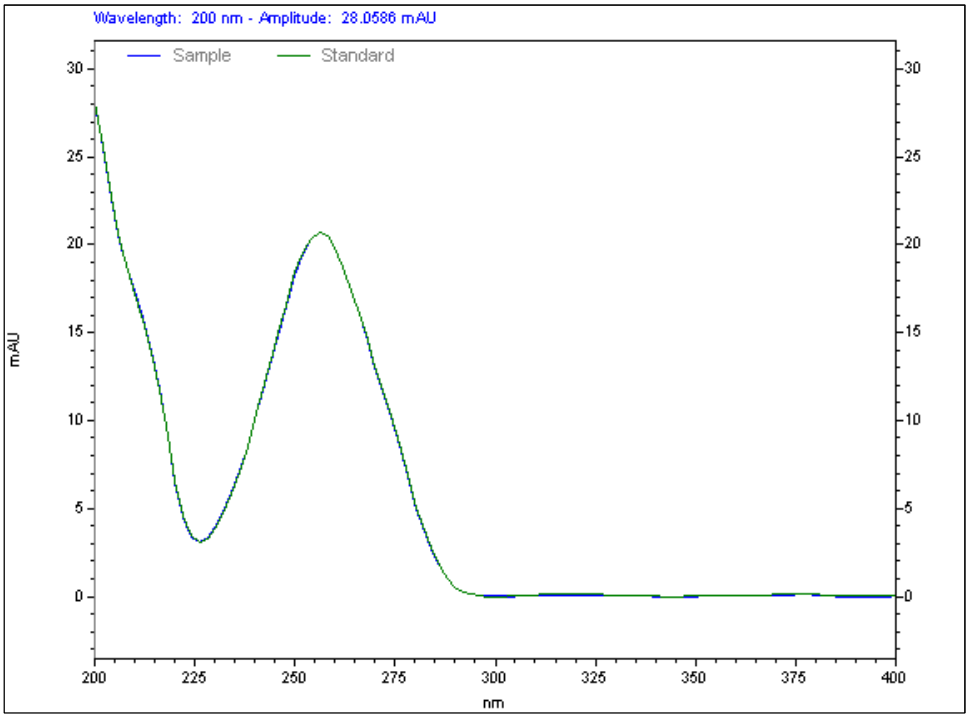


**Figure 25:** UV spectrum of the ethylparaben.

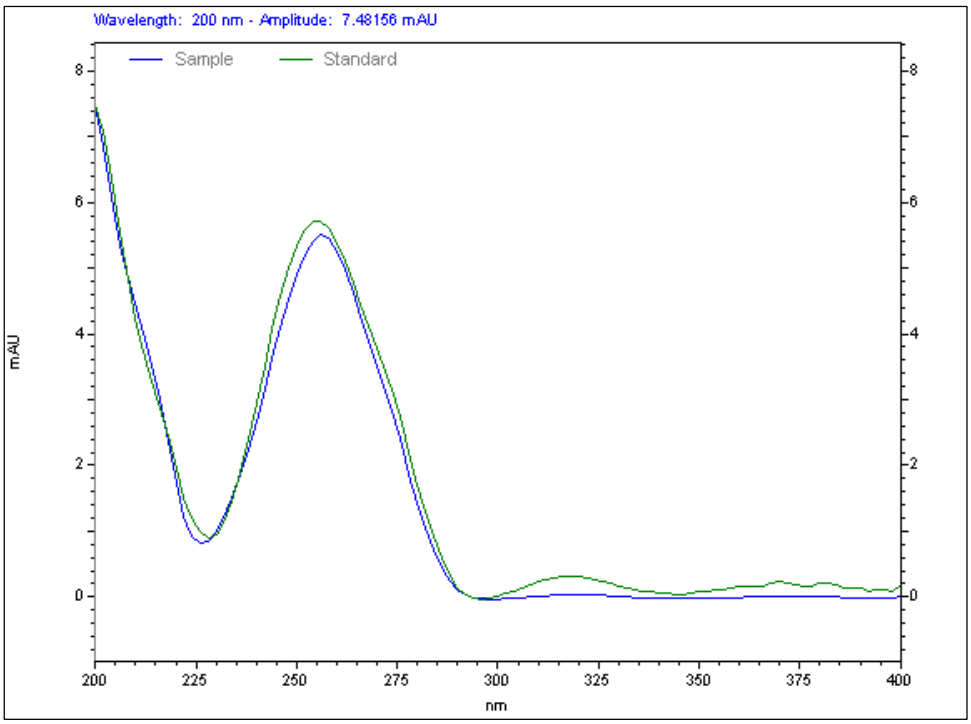


**Figure 26:** UV spectrum of the propylparaben.

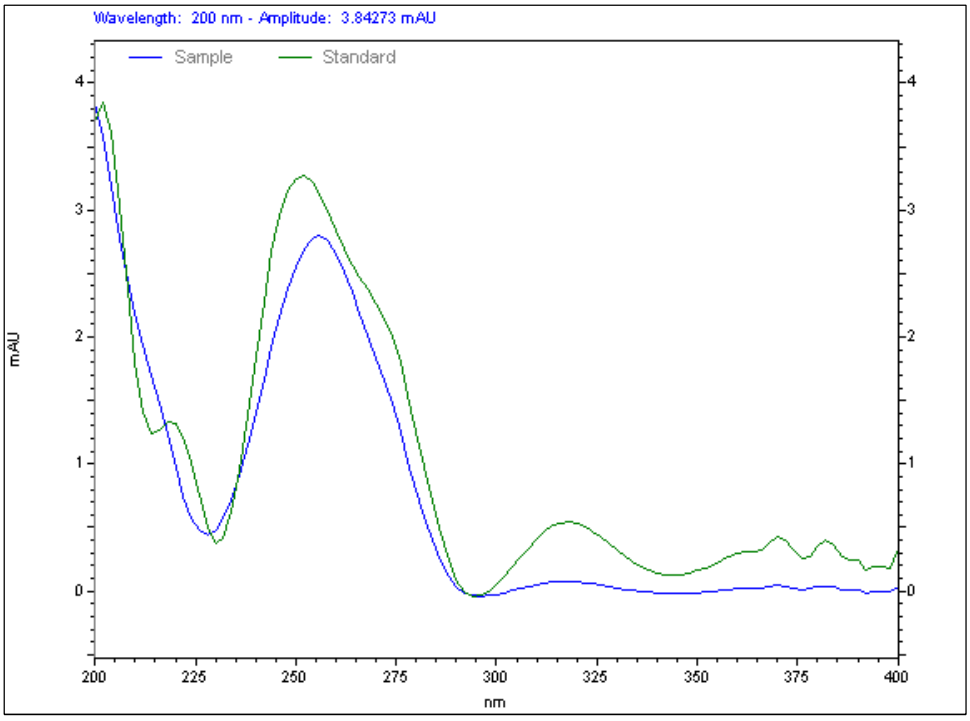


**Figure 27:** UV spectrum of the isobutylparaben.

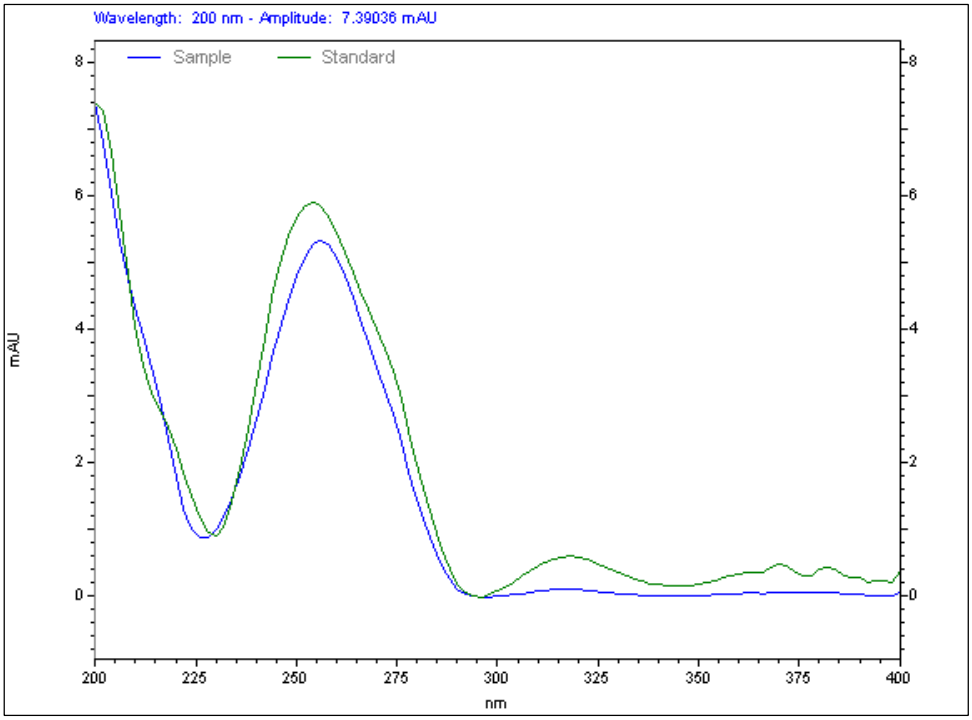


**Figure 28:** UV spectrum of the butylparaben.

**Table 34:** Similarity between *the working standard solution A* and *the Panthenol 4% gel sample*.

| Analyzed compound | Similarity |
|---|---|
| **Dexpanthenol** | 100.00 % |
| **Phenoxyethanol** | 100.00 % |
| **Methylparaben** | 100.00 % |
| **Ethylparaben** | 100.00 % |
| **Propylparaben** | 99.85 % |
| **Isobutylparaben** | 99.48 % |
| **Butylparaben** | 98.22 % |

Differences between the standard and the sample absorption curves are caused by very small amounts of the analyzed compounds in the samples/standards.

Retention times of all analyzed compounds were obtained from spiking *the Panthenol 4% gel sample* (see chromatograms Figures 29 and 30) in two different wavelengths, at 210 nm and 254 nm.

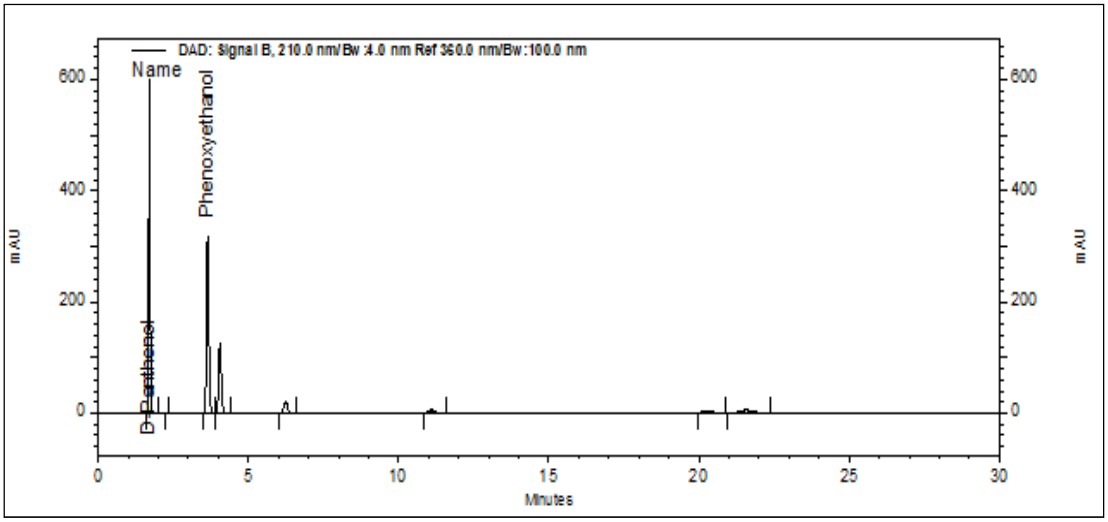


**Figure 29:** HPLC chromatogram of *the Panthenol 4% gel sample,* 210 nm.

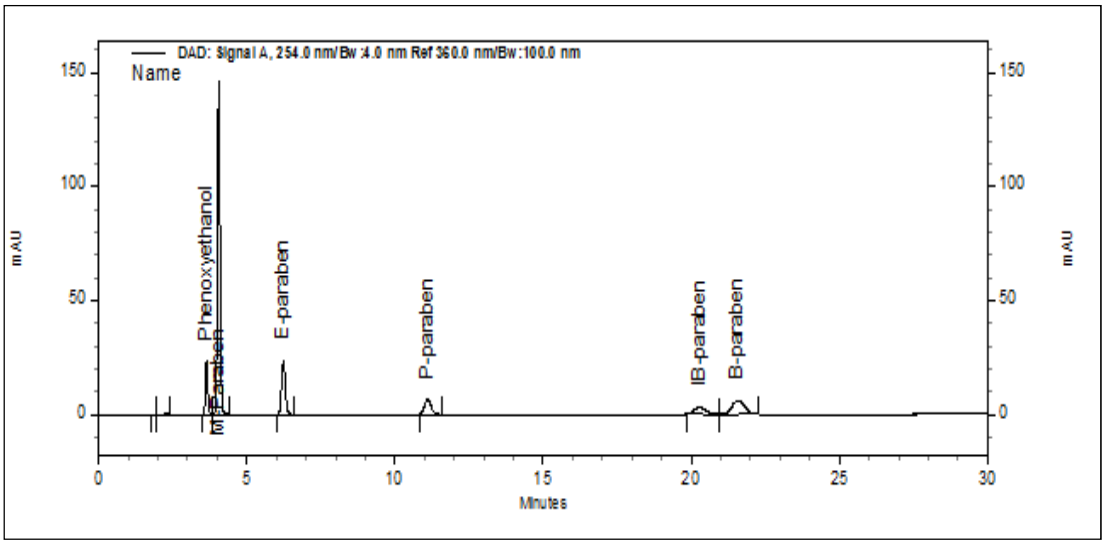


**Figure 30:** HPLC chromatogram of *the Panthenol 4% gel sample,* 254 nm.

**Table 35:** List of retention times.

| Analyzed standard | $t_R$ |
|---|---|
| **Dexpanthenol** | 1.700 min (± 5 %) |
| **Phenoxyethanol** | 3.650 min (± 5 %) |
| **Methylparaben** | 4.050 min (± 5 %) |
| **Ethylparaben** | 6.230 min (± 5 %) |
| **Propylparaben** | 11.100 min (± 5 %) |
| **Isobutylparaben** | 20.250 min (± 5 %) |
| **Butylparaben** | 21.550 min (± 5 %) |

## 5.5. Assay

The external standard method was selected as appropriate for the quantitative assay because of the high number of analyzed compounds. To determine the exact content of all compounds in *the Panthenol 4% gel sample* it was essential to compare parameters of *the working standard solution B* (known content of the compounds) and *the Panthenol 4% gel sample* (determined content of the compounds).

The chromatograms of the standards were obtained by injecting *the working standard solution B* six times under chromatographic system conditions; Table 37.

Two separate samples of *The Panthenol 4% gel sample* were prepared and injected two times each under chromatographic system conditions. The assay is calculated using the equation published in paragraph 3.6.2.2. (dilution factor Z=5). Final mean results of all analyzed compounds are presented in Table 38.

**Table 36:** Peak areas of *the working standard solution B*.

| Compound | Dexpanthenol | Phenoxyethanol | Methylparaben | Ethylparaben | Propylparaben | Isobutylparaben | Butylparaben |
|---|---|---|---|---|---|---|---|
| **Concentration (mg/100 ml)** | **100.91** | **14.12** | **3.1908** | **0.792** | **0.4084** | **0.4128** | **0.7968** |
| **F** | **0.996** | **0.9997** | **0.993** | **0.999** | **0.999** | **0.996** | **0.995** |
| No. 1 | 265281387 | 17177645 | 124636235 | 28700698 | 13473237 | 11681781 | 24343600 |
| No. 2 | 264489899 | 17085583 | 123783565 | 28413538 | 13359540 | 11614392 | 24006913 |
| No. 3 | 265244499 | 17130573 | 123798682 | 28422826 | 13386092 | 11604949 | 23947613 |
| No. 4 | 265537734 | 17154110 | 124001659 | 28487090 | 13364010 | 11651386 | 24154867 |
| No. 5 | 265929776 | 17198790 | 124191843 | 28543282 | 13375146 | 11655030 | 24211227 |
| No. 6 | 266364911 | 17210225 | 124138702 | 28535246 | 13420300 | 11634048 | 24035731 |
| **Mean** | **265474701** | **17159487.67** | **124091781** | **28517113.33** | **13396387.5** | **11640264.33** | **24116658.5** |

**Table 37:** Assay results.

| Compound | | **Dexpanthenol** | **Phenoxyethanol** | **Methylparaben** | **Ethylparaben** | **Propylparaben** | **Isobutylparaben** | **Butylparaben** |
|---|---|---|---|---|---|---|---|---|
| Sample No. 1 | Weight (mg) | 492.42 | 492.42 | 492.42 | 492.42 | 492.42 | 492.42 | 492.42 |
| | Peak Area No. 1 | 257294175 | 16751682 | 26577565 | 12021337 | 10376586 | 22308259 | 115699496 |
| | Peak Area No. 2 | 255894033 | 16669506 | 26470131 | 12032114 | 10356065 | 21635966 | 115224327 |
| | Mean | 256594104 | 16710594 | 26523848 | 12026725.5 | 10366325.5 | 21972112.5 | 115461911.5 |
| | **Result** | **3.945585** | **0.558324** | **0.029889** | **0.014877** | **0.014872** | **0.029337** | **0.119740** |
| Sample No. 2 | Weight (mg) | 506.93 | 506.93 | 506.93 | 506.93 | 506.93 | 506.93 | 506.93 |
| | Peak Area No. 1 | 265292281 | 17365173 | 27532310 | 12500166 | 10767830 | 23256481 | 119688868 |
| | Peak Area No. 2 | 265318601 | 17349263 | 27472338 | 12469877 | 10795363 | 23114243 | 119554741 |
| | Mean | 265305441 | 17357218 | 27502324 | 12485021.5 | 10781596.5 | 23185362 | 119621804.5 |
| | **Result** | **3.962767** | **0.563329** | **0.030105** | **0.015002** | **0.015025** | **0.030071** | **0.120503** |
| **Final Mean Result (%)** | | **3.954176** | **0.560827** | **0.120122** | **0.029997** | **0.014939** | **0.014948** | **0.029704** |
| Limit (100%) | | 4.000 | 0.576 | 0.128 | 0.032 | 0.016 | 0.016 | 0.032 |
| Interval (%) | | 3.8 – 4.2 | 0.5184 – 0.6336 | 0.1152 – 0.1408 | 0.0288 – 0.0352 | 0.0144 – 0.0176 | 0.0144 – 0.0176 | 0.0288 – 0.0352 |

In *the Panthenol 4% gel sample,* the mean concentration is:

3.9542 % of the dexpanthenol,

0.5608 % of the phenoxyethanol,

0.1201 % of the methylparaben,

0.0300 % of the ethylparaben,

0.0149 % of the propylparaben,

0.0149 % of the isobutylparaben,

and 0.0297 % of the butylparaben.

# Chapter 6 Conclusion

The conditions for the qualitative and quantitative determination of the dexpanthenol, and preservatives by high performance liquid chromatography coupled with diode-array detector were optimized and applied for the analysis of a topic gel based on acrylamide polymer.

Column Discovery $C_{18}$ (150 mm × 4.6 mm I.D., 5 μm) was selected as the most suitable stationary phase and potassium dihydrogen phosphate buffer and acetonitrile (67:33, v/v) was selected as the most effective composition of the mobile phase. The isocratic temperature mode of 25°C was used. The flow rate of the mobile phase was 1.00 ml $min^{-1}$ and injection volume of 5 μl was used. The detector was operated at two wavelengths (210 nm for the dexpanthenol and 254 nm for the phenoxyethanol, and the preservatives).

Three basic system suitability parameters were evaluated: the numbers of the theoretical plates ($N$) values of all compounds were greater than 6000 units, the determined symmetry factors of all compounds were in interval from 1.1 to 1.3, and the calculated values of resolution of peaks were greater than 1.5. The system suitability test meets the requirements of the Czech Pharmacopoeia. A few validation tests were performed. From the accuracy experiment, the method shows good recovery which is in the interval from 98.00 % to 101.00 % for all analyzed compounds. The RSD (%) is not greater than 0.40 % in the injection precision experiment and is not greater than 2.00 % in the method precision experiment. The intermediate precision of this chromatographic method performed statistically equivalent results. From the specificity and selectivity experiment, it is evident there was good separation of all compounds and visibly no interference in the elution zone of the analyzed compounds occurred. From the linearity experiment, the correlation coefficient was at least 0.99900 for all analyzed compounds and the calibration curves are linear in stated concentration levels.

This efficient method allows the simple and fast baseline separation of the dexpanthenol, the phenoxyethanol and the preservatives with good resolution in a single run.

# References


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# List of Figures

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# Abbreviations

| | |
|---|---|
| API | active pharmaceutical ingredient |
| $A_s$ | symmetry parameter |
| $A_{St}$ | peak area of standard |
| $A_x$ | peak area of sample |
| B-paraben | butylparaben |
| $c_{\%}$ | content of analyzed compound (%) |
| $c_0$ | concentration real (added) |
| $c_i$ | concentration determined (measured) |
| ČL09 | Czech Pharmacopoeia 2009 |
| d | baseline distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at 5% of the peak height |
| DAD | diode array detector |
| D-Panthenol | dexpathenol |
| E-paraben | ethylparaben |
| F | response factor of standard |
| FDA | Food and Drug Administration |
| FLD | fluorescence detector |
| GC | gas chromatography |
| GLC | gas-liquid chromatography |
| GLP | Good Laboratory Practice |
| HPLC | high-performance liquid chromatography |
| IB-paraben | isobutylpraben |
| ICH | the International Conference on Harmonisation |
| IEC | ion-exchange chromatography |
| LC | liquid chromatography |
| LLC | liquid-liquid chromatography |
| LSC | liquid-solid chromatography |
| M-paraben | methylparaben |
| $m_{St}$ | weighed amount of standard |
| $m_x$ | weighed amount of sample |

| | |
|---|---|
| N | number of theoretical plates |
| NMR | nuclear magnetic resonance |
| NPC | normal-phase chromatography |
| PC | paper chromatography |
| P-paraben | propylparaben |
| R (%) | recovery (%) |
| R | correlation coefficient |
| RDS | relative standard deviation (%) |
| RI | refractive index |
| $R_s$ | resolution |
| SD | standard deviation |
| SEC | size-exclusion chromatography |
| SPE | extraction chromatography |
| TLC | thin-layer chromatography |
| $t_R$ | retention time |
| $w_{0.05}$ | width at 5% of peak height measured from the baseline |
| $w_h$ | width of the peak of interest determined at half peak height |
| Z | dilution factor |
| $\lambda_{max}$ | maxima of absorption |