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SEPARATION OF PHTHALATES BY CYCLODEXTRIN MODIFIED MICELLAR ELECTROKINETIC CHROMATOGRAPHY. QUANTITATION IN PERFUMES.

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Abbreviations:

Ac, Corrected peak areas; Ac-β-CD, acetyl-β-CD; BGE, background electrolyte; BBP, benzyl butyl phthalate; CD, cyclodextrin; CD-MEKC, cyclodextrin modified micellar electrokinetic chromatography; CHES, n-cyclohexyl-2-aminoethanesulfonic acid; DAD, diode array detector; DAP, diallyl phthalate; DCP, dicyclohexyl phthalate; DBP, di-n-butyl phthalate, DEHP, diethyl

hexyl phthalate; DEP, diethyl phthalate; DiBP, diisobutyl phthalate; DM-β-CD, dimethyl-β-CD; DMP, dimethyl phthalate; DNPP, di-n-pentyl phthalate; DNOP, di-n-octyl phthalate; DPP, di-n-propyl phthalate; DPhP, diphenyl phthalate; EOF, electroosmotic flow; HP-β-CD, hydroxypropyl-β-CD; *k*, capacity factor; LOD, limit of detection; LOQ, limit of quantitation; Me-β-CD, methyl-β-CD; PVC, polyvinyl chloride plastics; SC, sodium cholate; SDC, sodium deoxycholate; SDS, sodium dodecyl sulfate; STDC, sodium taurodeoxycholate; STC, sodium taurocholate; SPE, solid phase extraction; TM-β-CD, trimethyl-β-CD.

Abstract

A new CE method has been developed for the simultaneous separation of a group of parent phthalates. Due to the neutral character of these compounds, the addition of several bile salts as surfactants (sodium cholate (SC), sodium deoxycholate (SDC), sodium taurodeoxycholate (STDC), sodium taurocholate (STC)) to the separation buffer was explored showing the high potential of SDC as pseudostationary phase. However, the resolution of all the phthalates was not achieved when employing only this bile salt as additive, being necessary the addition of neutral cyclodextrins (CD) and organic modifiers to the separation media. The optimized cyclodextrin modified micellar electrokinetic chromatography (CD-MEKC) method consisted of the employ of a background electrolyte (BGE) containing 25 mM β-CD-100 mM SDC in a 100 mM borate buffer (pH 8.5) with a 10 % (v/v) of acetonitrile, employing a voltage of 30 kV and a temperature of 25ºC. This separation medium enabled the total resolution of eight compounds and the partial resolution of two of the analytes, di-n-octyl phthalate (DNOP) and diethyl hexyl phthalate (DEHP) (Rs ~0.8), in only 12 min. The analytical characteristics of the developed method were studied showing their suitability for the determination of these compounds in commercial perfumes. In all the analyzed perfumes the most common phthalate was diethyl phthalate (DEP) that appeared in ten of the fifteen analyzed products. Also dimethyl phthalate (DMP), diallyl phthalate (DAP), dicyclohexyl phthalate (DCP), and di-n-pentyl phthalate (DNPP) were found in some of the analyzed samples.

Keywords: Phthalate; micellar electrokinetic chromatography; Bile salt, Cyclodextrin; Perfume.

1. Introduction

Phthalates are man-made chemicals produced worldwide in more than 1 million tons each year since 1920´s [1]. The term phthalate is referred to a class of chemicals derived from 1,2-benzenedicarboxylic acid that are dialkyl or alkylarylester substituted. The length of the alkyl chain determines the application field of the phthalate.

Phthalates with higher molecular weights, such as diethyl hexyl phthalate (DEHP) are commonly used as additives and plasticizers in polyvinyl chloride plastics (PVC). Approximately 93 % of all plasticizers employed in the world are phthalates and the remaining percentage corresponds to esters and polyesters based on adipate, phosphoric acid, sebacic acid, etc. [2]. Those phthalates with lower molecular weights such as diethyl phthalate (DEP) and dimethyl phthalate (DMP) are commonly used as solvents and odorless diluents in cosmetic products such as deodorants, hair products and perfumes [3, 4] and they are also used as additives in the textile industry and in pesticide formulation [5].

Phthalates have received special attention in the last years due to their ubiquitous presence in the environment [6], the clear evidences of their reproductive toxicity [7, 8], and their estrogenic activity [9]. The European Union has published a list of priority substances with a potential endocrine disrupting action, which includes di-n-butyl phthalate (DBP) and DEHP [10]. Moreover, they are also suspected of being carcinogenic, teratogenic, and mutagenic [11] being these evidences more strong for DEHP [12, 13]. Due to the fact that phthalates when employed in polymers, are not chemically bounded to the polymer, they can leach or outgas into the surrounding media and they are present in the environment in great amounts. On the other hand, although the toxicological information of phthalates is huge, there is little information about the pathways of human exposure to phthalates. However, their presence in milk and urine demonstrates the human exposure to these compounds [14]. Humans are exposed to phthalates in numerous ways, i.e., by

migration of phthalates into foodstuff, by dermal adsorption of phthalates from cosmetics, or by inhaling air containing them [15]. According to the US Environmental Protection Agency (EPA) phthalates such as DMP, DEP, DBP, benzyl butyl phthalate (BBP), DEHP, and di-n-octyl phthalate (DNOP) are listed as the priority pollutants among the phthalate esters [16].

In the field of their use in cosmetic products, the article 4 of the European directive 76/768/EEC, modified by the European directive 2004/93/CE, specifies the substances that due to their classification as carcinogenic, mutagenic or toxic to reproduction are forbidden in cosmetic products [17]. In this situation phthalates like DEHP, DBP, and BBP have been prohibited in cosmetics [17].

For all these reasons, there is a great interest in the development of new and rapid methods for the determination of parent phthalates in several matrices. The analysis of phthalates is mostly performed by GC because they are enough volatile and thermostable. The works reported in the literature concerning the separation and determination of phthalates by GC involve in general mass spectrometry detection as it has been reported by LaFleur and Schug [18]. Moreover the determination of phthalates by GC could involve a previous derivatization step that makes the sample preparation more tedious [19, 20]. However, the development of miniaturized approaches for the extraction, that can be easily coupled to GC, have resulted in more efficient sample enrichment. This is for example the case of solid phase microextraction (SPME) [21], dispersive liquid-liquid microextraction (DLLME) [22], etc. that have been successfully coupled to GC for the determination of phthalates. In recent years there is an increasing attention on the analysis of phthalic esters by HPLC and CE. HPLC is an especially interesting alternative for the analysis of isomeric mixtures of phthalates [23], and the employ of UPLC systems gives opportunities to improve chromatography in terms of separation, efficiency and detection limits due to the lower dilution of the sample [24]. Ultraviolet detection has been used for phthalate determination in several works [25] however, the use of MS has increased in recent years [26, 27]. On the other

hand, CE offers lower analysis times, lower consumption of reagents, higher efficiency and simplest methodology. There are several works concerning the separation of phthalates by CE in the literature [16, 28-33]. In all of them, due to the neutral character of these analytes, a charged pseudostationary phase is added to the separation buffer. In most works sodium dodecyl sulfate (SDS) is the added surfactant in order to give mobility to the analytes [16, 28, 29, 31-33]. However, in most of these works only the most hydrophilic phthalates were analyzed or no effective separation was achieved for those phthalates with higher octanol-water partition coefficients (i.e. DEHP and DNOP) [16]. Moreover in almost all the works reported the number of phthalates separated is lower than six. On the other hand bile salt monomers are more polar than SDS, and lead to a general reduction of capacity factor (*k)* values of hydrophobic compounds. Also bile salt micelles can tolerate a higher concentration of organic solvents that usually helps the separation [34]. In this sense Guo *et al.* [35] employed for the first time a bile salt as pseudostationary phase for the separation of six parent phthalates. In this work the employ of sodium cholate (SC) as pseudostationary phase allowed the separation of six phthalates but the analysis time achieved was around 40 min. Finally Sirimanne *et al.* [30] employed a C18 capillary column for capillary electrochromatography experiments achieving the separation of seven phthalates (DMP, DEP, diallyl phthalate (DAP), diphenyl phthalate (DPhP), BBP, DBP, and diisobutyl phthalate (DiBP)) in only 6.3 min. Furthermore it has to be noticed that the samples analyzed by CE were soil, serum and gunshot samples and that there is no work in the literature for the analysis of cosmetic samples by this separation technique.

The main problem when analyzing phthalates is the contamination that may result in false positive results. Due to the fact that phthalates are present in the whole analytical environment (gloves, adsorbed on glass, water, air, analytical equipment, etc.) all the material employed needs to be very carefully cleaned and all type of plastic materials must be avoided [36].

The aim of this work was to evaluate different pseudostationary phases (including bile salts and cyclodextrins) for the development of a rapid and simple CE method for the simultaneous separation of ten phthalates and their determination in perfume samples.

2. Materials and methods

2.1 Reagents and Samples

All reagents employed for the preparation of background electrolytes (BGEs) and samples were of analytical grade. Boric acid was supplied from Fluka (Buchs, Switzerland), sodium hydroxide from Merck (Darmstadt, Germany), methanol and acetonitrile were purchased from Scharlab (Barcelona, Spain), and n-cyclohexyl-2-aminoethanesulfonic acid (CHES) was from Sigma Aldrich (St. Louis, MO, USA).

β-CD, methyl-β-CD (Me-β-CD) (DS ~ 12), and trimethyl-β-CD (TM-β-CD) were supplied by Fluka, γ-CD, hydroxypropyl-β-CD (HP-β-CD) (DS ~ 3), and acetyl-β-CD (Ac-β-CD) (DS ~ 7) by Cyclolab (Budapest, Hungary) and dimethyl-β-CD (DM-β-CD) (DS~ 14-17) was supplied from Sigma Aldrich. Bile salts SC, sodium deoxycholate (SDC), sodium taurodeoxycholate (STDC) and sodium taurocholate (STC) were from Sigma Aldrich.

Standards of the phthalates, which structure is presented in **Figure 1**, DMP, DEP, DAP, DPP, DBP, DNPP, DCP, BBP, DEHP, and DNOP were supplied from Sigma. The perfumes were acquired in cosmetic shops in Alcalá de Henares (Madrid, Spain). A total amount of 15 perfume samples was analyzed.

The LC-C18 cartridges employed for clean-up of the samples were from Supelco (Bellefonte, PA, USA).

Water used to prepare all solutions was purified in a Milli-Q system from Millipore (Bedford, MA, USA).

2.2 Apparatus

A HP^{3D}CE system from Agilent Technologies (Palo Alto, CA, USA) with a diode array detector (DAD) was employed for the experiments. Instrument control and data acquisition were performed with the HP^{3D}CE ChemStation software. Separations were performed in an uncoated fused-silica capillary of 50 μm i.d. (375 μm o.d.) with a total length of 58.5 cm (50.0 cm to the detector) purchased from Polymicro Technologies (Phoenix, AZ, USA). UV detection was performed at 210 ± 2 nm, 240 ± 2 nm and 325 ± 2 nm. The UV detection wavelength selected for quantitation was 240 ± 2 nm, because although at this wavelength the absorption of phthalates is lower, there are less interferences than at 210 ± 2 nm and the signal to noise ratio (S/N) is higher. The wavelength 325 ± 2 nm was employed to identify interferences because at this wavelength phthalates do not absorb. A pH-meter model 744 from Metrohm (Herisau, Switzerland) was used to adjust the pH of the separation buffers. All the solutions were degassed in an ultrasonic bath Ultrasons-H from J.P. Selecta (Barcelona, Spain).

2.3 Glassware cleaning

Special care was taken to avoid the contact of reagents and solvents with plastic materials. All glassware was cleaned prior to the analysis according to the recommendations specified in the section 4.1.2 of U.S. EPA Method 506 [37]. All glassware was cleaned as soon as possible after its use by rinsing with the same solvent of the solution that was stored in the recipient. Next it was washed with hot water and detergent and rinsed with Milli-Q water. It was dried and heated in a muffle furnace at 400°C for one hour. After cooling, the glassware was sealed with aluminum foil and stored in a clean environment to prevent accumulation of dust and other contaminants.

2.4 Procedure

Before first use, the new capillary was rinsed with 1 M NaOH for 30 min, followed by 5 min with water and finally 60 min with the separation buffer at 25ºC. The capillary was rinsed between runs with 0.1 M NaOH for 2 min, water for 2 min, and BGE for 5 min. At the end of each day the capillary was rinsed with 5 min water, 5 min 0.1 M NaOH and 5 min water. The capillary ends were maintained during the night in Milli-Q water.

Running buffers were prepared by dissolving the appropriate amount of boric acid or CHES in Milli-Q water and adjusting the pH to the desired value with 1 M or 0.1 M NaOH. The final volume was adjusted by adding Milli-Q water to get the desired buffer concentration. BGEs were prepared by dissolving the appropriate amount of different CDs and bile salts in the running buffer containing the organic modifier selected in each experiment.

Stock standard solutions of parent phthalates were prepared by dissolving the appropriate amount of the compound in methanol up to a final concentration of 1000 mg L^{-1} and 10000 mg L^{-1}. To prepare the working solutions, different aliquots were diluted in methanol to obtain concentrations of each phthalate between 30 and 500 mg L^{-1} for the calibration by the external standard method. When standard addition calibration method was employed different amounts of standard solutions of phthalates were added to a commercial sample in the range 50-250 mg L^{-1}. For the optimization of the separation of the selected phthalates a standard solution containing each phthalate at 100 mg L^{-1} was employed.

All the standard solutions and BGEs were stored at 4ºC in the dark and they were filtered with a Nylon 0.45 µm pore size filter from Titan (Eatontown, NJ, USA) before their injection in the CE system.

To prepare the commercial formulations for their analysis, the method developed by Shen *et al.* [4] was followed. Briefly, 500 µL of perfume were transferred in a glass tube and 10 mL of methanol were added following by sonication during 30 min. After that, the sample was evaporated to dryness and redissolved in 25 mL 40 % (v/v) methanol. For clean-up of the sample solid phase

extraction (SPE) with a C18 cartridge was employed. The C18 cartridge was conditioned with 5 mL methanol, 5 mL water and 5 mL 40 % (v/v) methanol. The sample was loaded onto the column at a slow flow and after loading, the column was washed with 5 mL 40 % (v/v) methanol. Finally, phthalates were eluted with 5 mL of methanol and injected into the CE system.

2.5 Data treatment

The values of areas, migration times and resolution were obtained using the ChemStation software. For data treatment corrected peak areas (Ac) were used to compensate the differences in the electrophoretic conditions of each analyte and to obtain better reproducibility of data [38]. Limits of detection (LODs) and limits of quantitation (LOQs) were experimentally determined using the S/N ratio equal to 3 and 10, respectively [39].

The presence of matrix interferences was investigated by the comparison of the confidence interval of the slopes obtained when using the external standard calibration method and the standard additions calibration method. If the overlapping of the confidence intervals of the slopes of both calibration methods was demonstrated, no statistically significant differences between the slopes were obtained; hence the matrix did not produce systematic errors. The second method consisted on the employ of t-test for comparison of two calibration curves. If the p-value was up to 0.05 (for a confidence level of 95 %) it was considered that there were no significant differences between calibration curves.

Experimental data analysis and composition of graphs were carried out using Microsoft Office Excel 2007 and Origin 6.0 software.

3. Results and discussion

3.1 Evaluation of different bile salts as pseudostationary phases

Due to the fact that phthalates are neutral compounds, the addition of a pseudostationary phase that may interact with them is necessary in order to achieve their separation by CE. With the addition of an anionic surfactant, the phthalates can be separated on the basis of their relative affinity to the micellar environment. In this situation, the most hydrophobic compounds would be strongly associated to the micelles and will elute later while the most hydrophilic phthalates would elute earlier. As it has been mentioned into the introduction of this manuscript, bile salts offer several advantages over the most usual surfactants (i.e. SDS). These monomers are more polar than SDS, and lead to a general reduction of *k* values of hydrophobic compounds so they use to be more efficient in the separation of hydrophobic compounds as phthalates.

In this work, four bile salts were tested: SC, SDC, STC, and STDC at an initial concentration of 50 mM in 100 mM borate buffer (pH 8.5). A buffer at high pH was selected in order to obtain a high electroosmotic flow (EOF) that could move to the detector also the analytes that interact more strongly with the micelle. The other initial experimental conditions were as follows: uncoated fused-silica capillary, 50 μm x 58.5 cm (50.0 cm to the detector); temperature, 25°C; voltage, 25 kV; injection by pressure, 50 mbar x 2 s. When SC or STC were employed only the peaks corresponding to the less hydrophobic compounds were detected, thus DMP and DEP, and the other phthalates did not appear in the electropherogram in even 60 min of analysis. Thus, the interaction between the analytes and the bile salt was so strong that it was not possible to move the analytes towards the detector. These results could fit with those reported in the literature for bile salt SC [35], in which the analysis time was also quite long, although a less concentrated buffer was employed. For STDC bile salt, the first migrating peak was as expected DMP, that appeared at approximately 12 min and the last eluting peak, DNPP, migrated at 55 min, so a really long analysis time was achieved with this pseudostationary phase. Finally, when SDC was added to the separation media, the ten phthalates were analyzed in only 9 min, although as expected the separation of all of them was not achieved and all the compounds that migrated in the last part of the electropherogram,

thus those which interacted strongly with the surfactant, eluted together. With an initial concentration of 50 mM SDC added to the BGE it was achieved the complete separation of DMP, DEP, DAP, and DPP but the other six phthalates coeluted in only three peaks that were not completely resolved. As a consequence of the observed results, SDC was selected for further experiments.

To optimize the separation conditions for the selected phthalates, the concentration of SDC was varied from 25 to 100 mM in 100 mM borate buffer (pH 8.5). An increase in the SDC concentration resulted in a decrease of the EOF and thus all the phthalates migrated later. However, the increase in the concentration of the surfactant, resulted in less broadened peaks due to the fact that this additive increases the solubility of the analyzed compounds and the resolution between all the compounds was also improved (see supplementary material). Therefore a concentration of 100 mM SDC was chosen as the most adequate for further experiments. However, it has to be noticed that with an increasing concentration of SDC the situation of the separation achieved was quite similar to that obtained with 50 mM of SDC and only four of the phthalates were completely separated. The last eluting six compounds coeluted in only three peaks as it has been previously reported for 50 mM SDC.

3.2 Effect of the addition of different organic modifiers

Organic solvents such as methanol, acetonitrile or isopropanol, can be used as additives in the running buffer to improve the solubility of some analytes and also to cause a decrease in the EOF and thus an increase in the elution range. This influence has been studied in the literature and it has been proven that this is due to the changes in the dielectric properties of the electric double layer and of the charge generation on the fused-silica surface [40]. However, the concentration of organic modifier that can be added to the separation media in micellar electrokinetic chromatography

(MEKC) is limited because it can affect the formation of micelles. For this reason percentages below 20 % are usually employed, although bile salts can tolerate a higher concentration of organic solvents [34]. In this study, methanol and acetonitrile were added as organic modifiers to a separation media consisting of 100 mM SDC in 100 mM borate buffer (pH 8.5). At a percentage of 10 % (v/v) for both modifiers, similar resolution was achieved with them but longer migration times were obtained with methanol (~60 min) than with acetonitrile (~45 min) and this resulted in the broadening of the last eluting peaks (DBP, BBP, DCP, DNPP, DNOP and DEHP). For this reason, acetonitrile was selected as the most adequate organic modifier for the separation of the phthalates. However, the addition of acetonitrile to the BGE did not produce the total resolution of the ten phthalates and only nine peaks were observed.

3.3. Study of the addition of several neutral CDs to the BGE

In order to increase the selectivity against the studied compounds, the possibility of adding another pseudostationary phase to the BGE was explored. Due to the ionic character of the surfactants employed, the addition of several neutral CDs was tested. First of all, the addition of native β-CD and γ-CD at an initial concentration of 10 mM was investigated. Thus the separation media consisted of a 100 mM borate buffer (pH 8.5) containing 100 mM SDC, 10 mM of the CD and a 10 % (v/v) of acetonitrile. Under these conditions, phthalates can interact selectively with both pseudostationary phases and the separation could be improved. Only β-CD showed clear advantages in the separation of the selected compounds. With the addition of this CD to the BGE, the peaks eluting in positions seven and eight (DCP and DNPP) were slightly separated while till this moment they coeluted in a single peak and the total analysis time was around 15 min. When the surfactant was employed alone the analysis time was around 45 min and now with β-CD in the separation media the analysis time decreased drastically to only 15 min. The influence of the

concentration of β-CD was then investigated. **Figure 2** shows the effect of the concentration of β-CD added to the BGE in a range from 5 to 25 mM. As it can be observed, an increase in the concentration of β-CD resulted in a great increase in resolution, especially for the last six peaks (DBP, BBP, DCP, DNPP, DNOP and DEHP). With a concentration of 25 mM of β-CD all the studied phthalates were separated with resolutions between 3.1 and 25.6, except for the last peaks, corresponding to DNOP and DEHP, respectively, for which a resolution of 0.8 was achieved.

The employ of some derivatives from β-CD was also explored. The cyclodextrins Me-β-CD, DM-β-CD, HP-β-CD, TM-β-CD and Ac-β-CD were individually added at a concentration of 25 mM to the BGE containing 100 mM SDC dissolved in 100 mM borate buffer (pH 8.5) with 10 % (v/v) of acetonitrile. **Figure 3** shows the electropherograms obtained when each CD was added to the separation media. As it can be observed, with Me-β-CD, HP-β-CD, and Ac-β-CD the separation achieved was very similar to that obtained with the native CD, thus all the peaks were resolved except DNOP and DEHP. TM-β-CD did not offer any advantage over the others because with this CD the separation of the peaks corresponding to DBP and BBP was lost. Finally, with DM-β-CD the separation was not good in general but it was able to baseline separate DNOP and DEHP. For this reason it was thought that maybe the mixture of β-CD and DM-β-CD could be the solution to achieve a baseline resolution for all the analytes. Thus, the simultaneous addition of both CDs to the BGE at a concentration of 25 mM for each one was evaluated. However, in this proportion the total resolution of DNOP and DEHP was achieved but for the peaks corresponding to DBP, BBP, DCP and DNPP the resolution was completely lost. If the concentration of DM-β-CD was decreased to 30 mM, the resolution of DNOP and DEHP was lost so no advantage was observed compared with the employ of β-CD alone and if the concentration of DM-β-CD was increased the resolution of DBP, BBP, DCP and DNPP was completely lost. Finally when the concentration of DM-β-CD was decreased to 15 mM maintaining the concentration of β-CD constant at 25 mM, it was observed also a lost on resolution for DBP and BBP that coelluted in one peak. For this reason, only β-CD

was employed in the separation buffer although it did not enable the complete resolution of DNOP and DEHP. In conclusion, β-CD was selected as the second pseudostationary phase at a concentration of 25 mM added to the BGE containing 100 mM SDC in 100 mM borate buffer (pH 8.5) with 10 % (v/v) of acetonitrile.

The addition of different percentages of acetonitrile to the BGE was next investigated from 5 to 15 % (v/v) in order to observe its influence at higher and lower proportions of organic modifier than 10 % (v/v). While a lower percentage resulted in the complete lost of baseline resolution for all compounds except for those migrating in the first four positions (DMP, DEP, DAP, DPP), an increase of acetonitrile from 10 to 15 % (v/v) did not have any benefit in terms of resolution and moreover longer analysis times were achieved. For this reason, a percentage of 10 % (v/v) of acetonitrile was chosen.

3.4 Effect of the separation voltage and buffer nature

Some further experiments were performed in order to decrease the analysis time. The first attempt consisted of increasing the voltage applied for the separation from 25 kV to 30 kV. This change resulted in very similar resolutions than those obtained with 25 kV but the analysis time was shortened in more than 5 min. For this reason a separation voltage of 30 kV was selected. Finally, the employ of an organic buffer instead of 100 mM borate (pH 8.5) was studied. A 100 mM CHES buffer (pH 10.0) was selected because on one hand an organic buffer may help to dissolve better the analytes and consequently better resolution could be obtained and in the other hand a higher pH is supposed to reduce the migration time of analytes. Surprisingly this buffer did not improve the resolution of the studied phthalates and the analysis times were longer than with borate (20 and 12 min, respectively).

In conclusion, the final conditions selected for the simultaneous separation of DMP, DEP, DAP, DPP, DBP, BBP, DCP, DNPP, DNOP, and DEHP were: uncoated fused-silica capillary, 50 μm x 50.0 cm (t.l. 58.5 cm); BGE: 25 mM β-CD 100 mM SDC in 100 mM borate buffer (pH 8.5) containing a 10 % (v/v) of acetonitrile; temperature, 25°C; voltage, 30 kV; injection by pressure, 50 mbar x 2 s. Under these conditions, the baseline separation of all compounds except DNOP and DEHP, that were only resolved with a resolution of 0.8, was possible. However, since DNOP is not usually present in cosmetic samples, the developed method was applied to the determination of the other nine phthalates in commercial perfume samples.

3.5 Quantitative analysis of selected phthalates in commercial perfumes

Before carrying out the quantitative determination of DMP, DEP, DAP, DPP, DBP, BBP, DCP, DNPP, and DEHP in perfume samples, the analytical characteristics of the method were evaluated in terms of linearity, LODs, LOQs, precision, accuracy and selectivity. The results obtained are grouped in **Table 1.**

Linearity was determined by plotting Ac as a function of the concentration of each compound in the range 30-500 mg L^{-1}. A total number of seven standard solutions were individually prepared and injected by triplicate. This process was repeated during three different daysin order to check the repeatability of the method and to fix the linear range for each compound. **Table 1** presents this interval, the linear equation obtained in the selected range as well as the standard errors for the intercept (S_a) and the slope (S_b), and the determination coefficient (R^2). Satisfactory results were obtained in terms of linearity with $R^2 > 0.98$.

LODs and LOQs for the nine compounds were experimentally determined using a S/N ratio equal to 3 and 10, respectively. LODs values were between 7.1 and 19.2 mg L^{-1} and LOQs between 21.4 and 57.7 mg L^{-1} for the nine analyzed phthalates, as it can be observed in **Table 1**.

Precision of the methods was evaluated as *instrumental repeatability* and *intermediate precision*. Instrumental repeatability was determined from six repeated injections of a standard solution at two different concentration values of each compound (50 and 200 mg L^{-1}). The RSD values (%) obtained (**Table 1**) were lower than 1.6 % for migration times and lower than 9.7 % for Ac for both concentration levels. Intermediate precision was assessed at the same concentration levels for three consecutive days injecting each sample by triplicate each day. As it can be observed in **Table 1** the RSD values achieved were under 2.5 % and 11.6 % for analysis times and Ac respectively.

The selectivity of the method was demonstrated due to the absence of matrix interferences. For this purpose the slopes of the calibration lines obtained by the external calibration method and the standard additions calibration method were compared for two selected perfume samples (perfumes H and L). These two samples were selected for the study of matrix interferences because they showed the most complex matrix in preliminary experiments. The standard additions calibration line was obtained by spiking the diluted perfumes with known concentrations of a mixture of nine phthalates in the linear interval established for them (+0 mg L^{-1}, +50 mg L^{-1}, + 100 mg L^{-1}, +200 mg L^{-1}, +250 mg L^{-1}). The comparison of the confidence limits of the slopes obtained by each calibration method for each compound showed that there were no statistically significant differences between the slopes obtained by each calibration method for every compound. The results were confirmed by p-value of t-test and as it can be observed in **Table 1** the p-values obtained for all the compounds were above 0.05 at a confidence level of 95 %, demonstrating again the suitability of external calibration method for the quantitation of all the phthalates in the selected samples.

Accuracy of the method was evaluated as the recovery percentage obtained for all the analytes when a commercial perfume was spiked with known concentrations of each compound and subjected to the extraction procedure. For this purpose a perfume (one of those that did not present phthalates, perfume J) was selected and it was spiked with the standards of each phthalate in order

412 to obtain a concentration of 200 mg L^{-1} and 50 mg L^{-1} in the final extract. Mean recovery values
413 obtained were between 68 and 114 % as it is presented in **Table 1**.

414 The developed method was applied to the determination of these phthalates in fifteen perfumes.
415 **Figure 4** shows the electropherograms obtained for a standard solution containing each phthalate at
416 a concentration of 100 mg L^{-1} and several perfume samples after SPE with C18 cartridges. The
417 experimental conditions consisted of uncoated fused-silica capillary, 50 μm x 58.5 cm (50.0 cm to
418 the detector); BGE: 25 mM β-CD-100 mM SDC in 100 mM borate buffer (pH 8.5) containing a 10
419 % (v/v) of acetonitrile; temperature, 25°C; voltage, 30 kV; injection by pressure, 50 mbar x 2 s. As
420 it can be observed in this figure, the perfumes A and I contained two phthalates each one. The
421 phthalates present in perfume A were found to be DMP and DAP and for perfume I the phthalates
422 found were DEP and DCP. On the other hand the perfumes M and H presented three phthalates
423 each one which corresponded to DMP, DEP and DCP for perfume M and to DMP, DEP and DNPP
424 for perfume H. Finally the perfume J did not show any of the studied phthalates. The determined
425 amounts in the analyzed perfumes are specified in **Table 2.** As it can be observed in **Table 2**, eleven
426 of the analyzed perfumes presented at least one of the studied phthalates and only four of the
427 samples did not contain any of the selected analytes. The founded phthalates corresponded to DMP,
428 DEP, DAP, DNPP and DCP. It has to be highlighted that in none of the samples the phthalates
429 prohibited in cosmetic products were found, that is DEHP, DBP and BBP [17]. The most frequently
430 found phthalate in these cosmetic products was DEP, as it has already been proved in previous
431 works [4, 41]. This phthalate appeared in ten of the analyzed perfumes, that is in all the perfumes
432 containing phthalates except of one (perfume A), in the concentration range between 76 and 3115
433 mg L^{-1}. Regarding DAP and DNPP, each of these phthalates was only found in one perfume
434 (perfume A and perfume H, respectively) while DMP and DCP were detected in three perfumes
435 each one.
436

4. Concluding remarks

A new CD-MEKC methodology employing SDC and β-CD as pseudostationary phases has been developed in this work. The new method is able to separate ten phthalates (DMP, DEP, DAP, DPP, DBP, DNPP, DCP, BBP, DNOP, and DEHP) in only 12 min with resolutions above 3.1 for all the compounds, except of DEHP and DNOP for which a resolution of 0.8 was achieved.

Compared with the scarce methodologies reported in the literature concerning the simultaneous separation of phthalates by CE, this method employs for the first time SDC as pseudostationary phase. As commented before, there is only one work in the literature employing a bile salt as pseudostationary phase (SC) but the analysis times achieved were around 40 min, quite long compared with that obtained in the present work and considering that it presented only the separation of six parent phthalates. In general, it can be assessed that the present work improves the total analysis time (is of only 12 min) of all the previous works in the literature by this separation technique. In fact, there is only one work that separates as many analytes as presented here, the analysis time is around 58 min and it is not achieved the separation of DNOP and DEHP that coelute in a single peak. However, the present work achieves the separation of the most hydrophobic phthalates DEHP and DNOP, and although it is not achieved their baseline separation, it is achieved a resolution of 0.8 that is enough to distinguish between the two compounds in the real samples.

The developed method was validated in terms of linearity, precision, accuracy, LODs, and LOQs and after assessing its suitability it was applied to the quantitation of selected phthalates in perfume samples. The most common phthalate in the analyzed perfumes was DEP that appeared in ten of the selected perfumes. From the other phthalates only DMP, DAP, DCP, and DNPP were found in some of the analyzed samples.

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536

537 **Figure Captions**

538 **Figure 1:** Structures of the selected parent phthalates**.**

539 **Figure 2:** Separation by CD-MEKC of the selected phthalates using concentrations of β-CD
540 between 5 and 25 mM. Other experimental conditions: uncoated fused-silica capillary, 50 μm x
541 58.5 cm (50.0 cm to the detector); BGE: 100 mM SDC in 100 mM borate buffer (pH 8.5)
542 containing a 10 % (v/v) of acetonitrile; temperature, 25°C; voltage, 25 kV; injection by pressure, 50
543 mbar x 2 s.

544 **Figure 3:** Separation by CD-MEKC of the selected phthalates using different neutral cyclodextrins
545 (β-CD, Ac-β-CD, Me-β-CD, DM-β-CD, TM-β-CD and HP-β-CD) at a concentration of 25 mM.
546 Other experimental conditions as in Figure 2.

547 **Figure 4:** Electropherograms corresponding to the separation of selected phthalates by CD-MEKC
548 for an standard solution of 100 mg L^{-1} and five perfumes extracted by SPE according to the
549 procedure explained in 2.4 section. Experimental conditions: BGE: 25 mM β-CD-100 mM SDC in
550 100 mM borate buffer (pH 8.5) containing a 10 % (v/v) of acetonitrile; voltage, 30 kV. Other
551 experimental conditions as in Figure 2.

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Table 1: Analytical characteristics of the developed method for the separation of parent phthalates

Analytical characteristics	DMP		DEP		DAP		DPP		DBP		DNPP		DCP		BBP		DEHP	
Precision (RSD)																		
Concentration level (mg L^{-1})	50	200	50	200	50	200	50	200	50	200	50	200	50	200	50	200	50	200
Instrumental repeatability																		
Ac, RSD (%)	7.91	3.67	8.04	6.36	5.52	5.74	6.44	3.44	9.73	3.83	9.21	6.59	3.15	4.94	8.64	5.70	2.59	5.83
t, RSD (%)	0.56	0.36	0.57	0.40	0.60	0.37	0.64	0.39	0.76	0.38	1.05	1.11	0.81	0.92	0.76	0.70	1.34	1.56
Intermediate precision																		
Ac, RSD (%)	8.94	9.19	10.0	9.52	11.6	10.7	7.30	8.65	8.70	10.3	11.3	6.43	10.1	8.28	7.96	9.48	8.60	11.2
t, RSD (%)	1.35	1.37	1.53	1.87	1.58	2.28	1.58	2.51	1.47	2.34	1.68	1.72	1.52	2.33	1.47	2.47	1.78	1.29
Linearity																		
Linear range (mg L^{-1})	50-300		50-300		50-300		50-300		50-300		50-300		50-300		50-300		50-300	
Linear equation (bx + a)	0.0129 x + 0.0107		0.0097x + 0.1668		0.0077x + 0.2207		0.0072x + 0.2297		0.0059 x + 0.1119		0.0047x + 0.0898		0.0033x + 0.1131		0.0051x + 0.1006		0.0026x + 0.0352	
Standard errors	Sb=0.0003		Sb=0.0003		Sb=0.0005		Sb=0.0006		Sb=0.0003		Sb=0.0002		Sb=0.0003		Sb=0.0003		Sb=0.0001	
	Sa=0.0606		Sa=0.0561		Sa=0.0874		Sa=0.1010		Sa=0.0564		Sa=0.0448		Sa=0.0532		Sa=0.0568		Sa=0.0264	
Determination coefficient (R^2)	0.9978		0.9966		0.9871		0.9803		0.9907		0.9946		0.9852		0.9874		0.9942	
Accuracy (50 mg L^{-1})																		
Median Recovery (%)	68 ± 6		88 ± 6		114 ± 10		105 ± 6		113 ± 9		100 ± 8		104 ± 2		110 ± 13		91 ± 8	
LOD (mg L^{-1})	8.6		8.6		7.6		7.8		11.4		7.1		15.6		10		19.2	
LOQ (mg L^{-1})	25.9		25.9		22.7		23.4		34.2		21.4		46.8		30		57.7	
Study of matrix																		
(b ± t· Sb/vn)																		
External calibration	0.0129 ± 0.0006		0.0097 ± 0.0005		0.0077 ± 0.0008		0.0072 ± 0.0009		0.0059 ± 0.0005		0.0047 ± 0.0004		0.0033 ± 0.0005		0.0051 ± 0.0005		0.0026 ± 0.0002	
Standard addition	0.0142 ± 0.0023		0.0140 ± 0.0026		0.0089 ± 0.0017		0.0074 ± 0.0006		0.0046 ± 0.0006		0.0048 ± 0.0005		0.0034 ± 0.00006		0.0047 ± 0.0028		0.0029 ± 0.0003	
p-value	0.09035		0.0653		0.0699		0.0616		0.1381		0.1531		0.0802		0.1194		0.2363	

Table 2: Determined contents (mg L^{-1}) of analyzed phthalates in commercial perfumes (average value ± SD) (n=3). DPP, DBP, BBP and DEHP were not detected in any perfume.

Perfume Sample	DMP	DEP	DAP	DNPP	DCP
A	787 ± 54	n.d.	520 ± 67	n.d.	n.d.
B	n.d.	1665 ± 186	n.d.	n.d.	n.d.
C	n.d.	n.d.	n.d.	n.d.	n.d.
D	n.d.	1536 ± 73	n.d.	n.d.	n.d.
E	n.d.	769 ± 58	n.d.	n.d.	n.d.
F	n.d.	n.d.	n.d.	n.d.	n.d.
G	n.d.	n.d.	n.d.	n.d.	n.d.
H	446 ± 21	1655 ± 98	n.d.	331 ± 15	n.d.
I	n.d.	1721 ± 145	n.d.	n.d.	<LOQ
J	n.d.	n.d.	n.d.	n.d.	n.d.
K	n.d.	1210 ± 38	n.d.	n.d.	n.d.
L	n.d.	477 ± 36	n.d.	n.d.	557 ± 49
M	1207 ± 43	3115 ± 167	n.d.	n.d.	1496 ± 89
N	n.d.	2021 ± 228	n.d.	n.d.	n.d.
O	n.d.	76 ± 13	n.d.	n.d.	n.d.

Figure 1

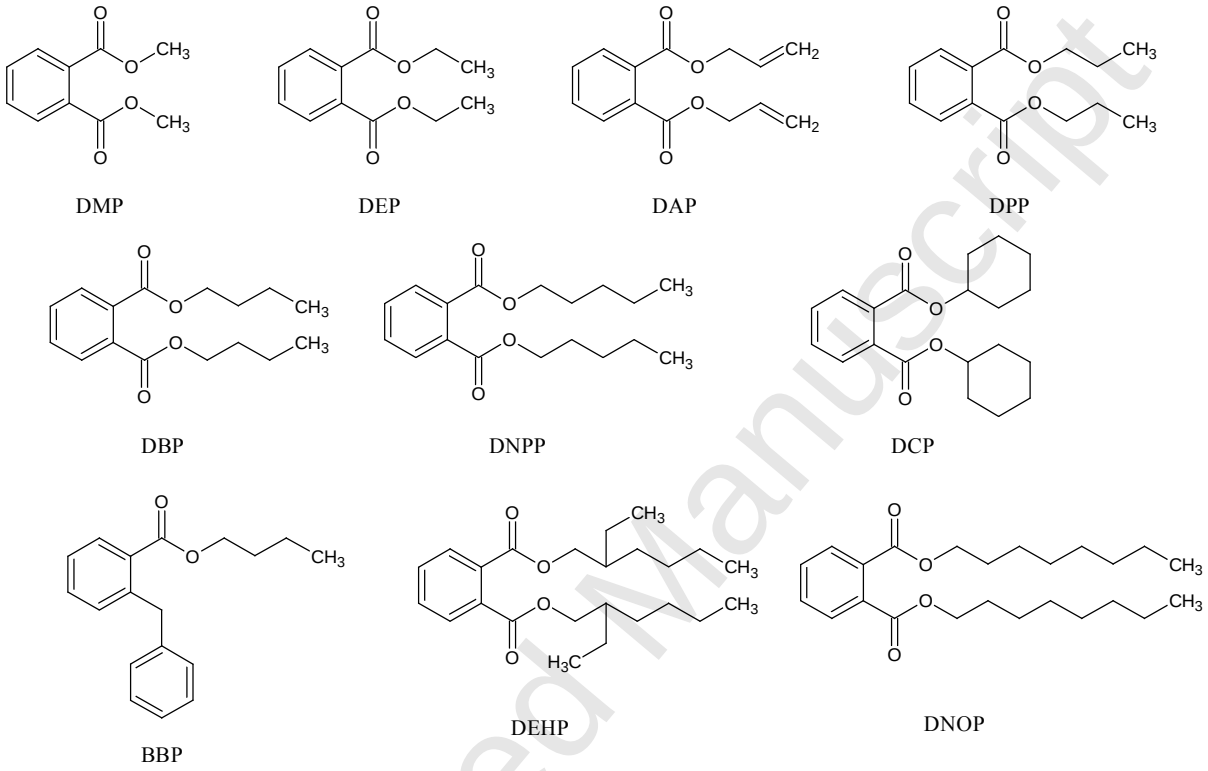


Figure 2

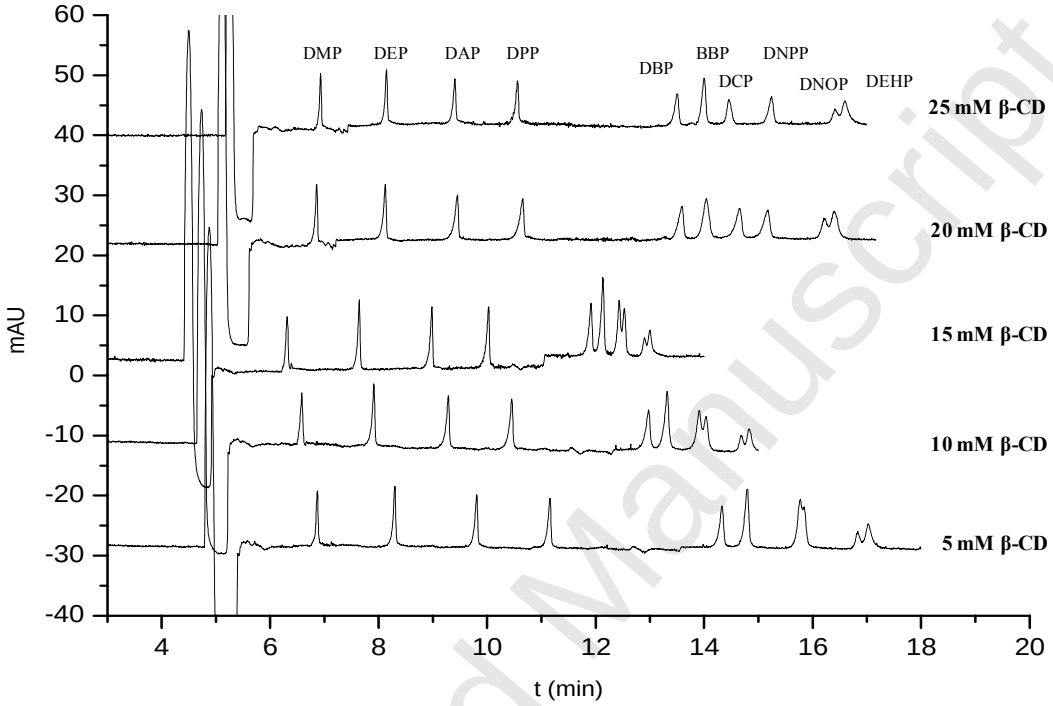


Figure 3

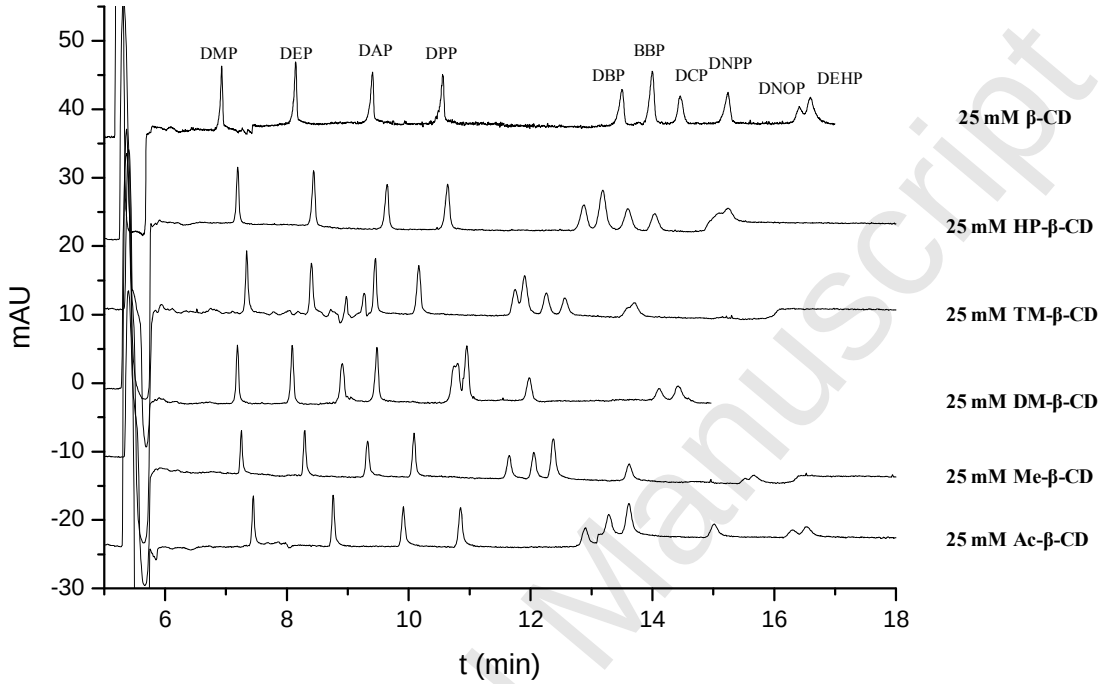
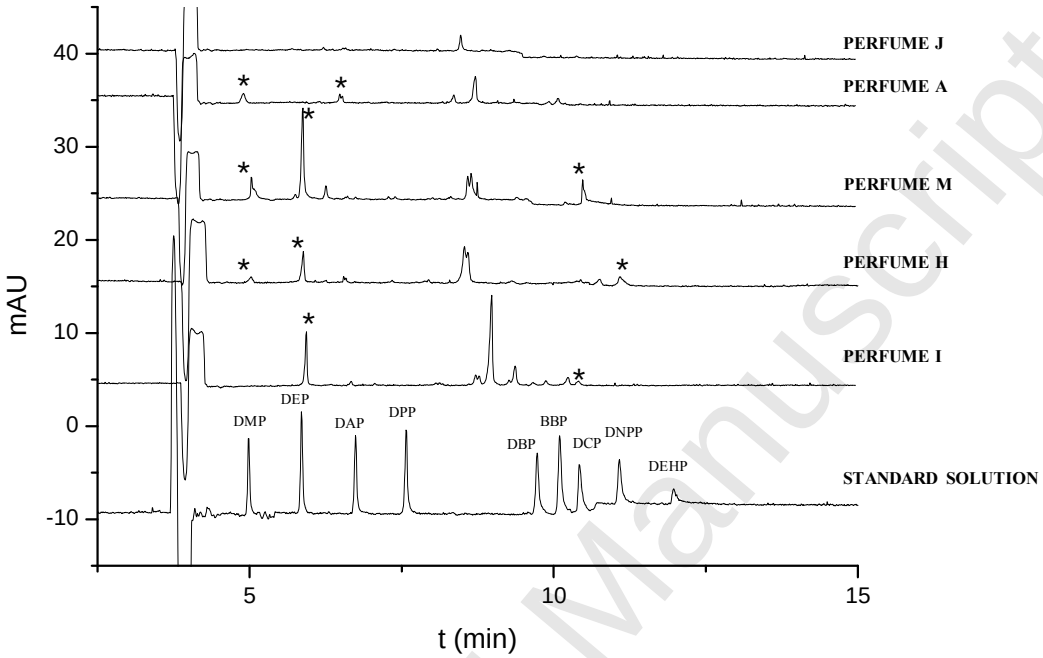


Figure 4



Highlights

- A new method by CD-MEKC has been developed for the analysis of phthalates.
- Simultaneous separation of ten phthalates has been achieved.
- The analytical characterisation of the method is satisfactory.
- The method is successfully applied to the determination of phthalates in perfumes.