Modulation of retention and selectivity in oil-in-water microemulsion liquid chromatography: A review

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Abstract

Microemulsions (MEs) are stable, isotropically clear solutions consisting of an oil and water stabilized by a surfactant and a co-surfactant. Microemulsion liquid chromatography (MELC) is a relatively new chromatographic mode, which uses an oil-in-water (O/W) ME as mobile phase. Retention, selectivity and efficiency can be modified by changing the concentration of the ME components and the ratio between the aqueous and oil phases. This work makes a critical survey on the information found in the literature about the mobile phase compositions that lead to the creation of successful O/W ME mobile phases, as well as the effect of pH for ionizable compounds and temperature. The viability of performing the analyses using isocratic and gradient elution is also considered. The complexity of the composition of a successful ME, and the fact that the different factors interact each other, may require many manipulations during method development to achieve an acceptable separation for complex mixtures. This is the reason of the proposal from several authors of a standard ME as starting point when developing a method for a new separation with no previous reports. Based on these initial conditions, the interest of several authors in applying computer-assisted approaches to optimize the composition of ME mobile phases, and reduce significantly the time and reagent consumption for method development, is described. Some practical tips are given to prepare stable ME mobile phases that yield reproducible results.

Keywords: Microemulsion liquid chromatography; Oil-in-water microemulsions; Experimental factors; Modulation of selectivity; Optimization of resolution

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1. Introduction

Microemulsions (MEs) are thermodynamically stable, transparent, optically clear, and isotropic liquid dispersions comprising oil phase, surfactant, co-surfactant (usually a medium chain-length alcohol), and water phase. Both hydrophilic and hydrophobic compounds can be dissolved in MEs, which are extensively applied in different fields. MEs have been used as pseudostationary phases in capillary electrophoresis, and as mobile phases in high-performance liquid chromatography (HPLC), in techniques called microemulsion electrokinetic chromatography (MEEKC) [1], and microemulsion liquid chromatography (MELC) [2–5], respectively.

There are three types of MEs: oil-in-water (O/W), water-in-oil (W/O), and a continuous phase with a sponge-like structure containing a mixture of the two liquids [6,7]. The formation of each type depends on the temperature, and nature and concentration of the components. However, in HPLC, O/W MEs are mostly used combined with conventional reversed-phase liquid chromatography (RPLC) stationary phases, due to their high water content, low viscosity and solubilizing power, and certainly, the popularity of RPLC. In MELC, solutes are partitioned between ME droplets, the stationary phase modified by adsorption of surfactant and co-surfactant, and bulk solvent (Fig. 1). Separations are usually carried out using isocratic elution.

MELC has been developed based on the principles of micellar liquid chromatography (MLC), where the mobile phase is composed of micellar solutions [9,10]. An O/W ME is, in fact, a modification of a micellar system where a lipophilic organic solvent is incorporated inside the micelles. Since the eighties, several hundreds of successful reports have been published in MLC. However, the analysis of samples containing highly non-polar compounds has been a problem. Some authors thought MELC could be the solution. MELC presents unique selectivity and several advantages compared to MLC and conventional RPLC, for the analysis of water-insoluble species, even in mixtures with hydrophilic compounds, owing to

the more complex interactions with solutes expected in MELC. The reduced retention times, equivalent or superior efficiency with implications in the resolution, and unique solubilizing power of MEs, are the most remarkable features of MELC.

Although the first report on O/W MELC appeared in 1992 [11], major interest in this chromatographic mode began only after 2003. There are three early reviews published in 2005 [2], 2007 [3], and 2008 [4]. Recently, we were interested in developing procedures in MELC, but found that the big amount of published information should be revised thoroughly, and especially, needed to be organized. We first published a review that analyzes the mechanisms of retention in MELC with O/W MEs, and describes the great variety of reagents suitable to act as ME oils, surfactants and co-surfactants. The published analytical procedures were also summarized [5]. Most applications in MELC refer to the analysis of hydrophobic compounds, both in pharmaceuticals, physiological fluids and other biological materials. The methods include the analysis of alkaloids, antihypertensive agents, cholesterol lowering drugs, steroids, tetracycline antibiotics, and vitamins, among other compounds. The number of reports is still limited, but MELC seems promising, and nowadays it is receiving growing attention.

Simple variation in the polarity of the internal phase (typically, heptane, octane, cyclohexane, diisopropyl ether, and ethyl acetate), as long as changes in the nature of surfactants (mainly sodium dodecyl sulfate, SDS, and polyoxyethylene(23)lauryl ether also known as Brij-35), and co-surfactants (mainly 1-propanol, 1-butanol, and 1-pentanol), affect the absolute and relative retention of analytes. It is noteworthy that in almost every MELC report, the concentration ranges of the ME reagents are systematically investigated. We found, however, an enormous variability of experimental conditions, which should be inspected in detail to be useful. The discussion of the modulation of retention, selectivity and efficiency by varying the concentration of the reagents (oil, surfactant and co-surfactant) that form a ME, as well as the ratio between the aqueous and oil phases, needed a dedicated work. In this review,

we give some orientations on the compositions leading to the creation of successful O/W ME mobile phases. The final choice will depend on the required separation speed, selectivity and efficiency. The viability of performing the analyses using isocratic and gradient elution, as well as the optimization of mobile phase composition, is also commented.

2. Concentration ranges of the microemulsion reagents in MELC

Changes in the ME reagents (nature and concentration) affect not only the formation of stable oil droplets and elution strength, but also the stationary phase nature, since oil, surfactant and co-surfactant molecules all can be adsorbed onto the stationary phase [11,12]. Any variation in the ME composition can alter the equilibrium between the two phases, and consequently, the chromatographic behavior of solutes in MELC. With a proper selection of the concentrations of surfactant and co-surfactant, the ME will be able to solubilize a greater proportion of oil. This in turn will enable the development of procedures suitable for the analysis of particularly water-insoluble compounds [13]. It is, thus, very important to select a proper mobile phase composition with regard to all ME components, in order to achieve satisfactory separations.

The complex nature of MEs yields numerous composition options for getting good separation performance, when compared to other chromatographic modes. The differences in the types and concentrations of oil, surfactant and co-surfactant could result in a great diversity of results, as will be next shown.

2.1. Concentration of surfactant

The critical micellar concentration (CMC) is the concentration of surfactant molecules in solution at which micelles start to form. Further addition of surfactant will increase the number of micelles, while the amount of free surfactant molecules in solution will remain

constant. The CMC values in aqueous medium for SDS and Brij-35, the most usual surfactants in reported MELC methods, are 8.2 mM and 0.06 mM, respectively [9].

When an organic solvent is added to the aqueous medium, the CMC values are altered. A study carried out for SDS micellar mobile phases, in the presence of different co-surfactants at increasing concentration, is very illustrative in this regard [14]. The CMC of SDS increased with methanol and acetonitrile, whereas it decreased with 1-propanol, 1-butanol and 1-pentanol. For 1-butanol and 1-pentanol, which partition into the micelle, the CMC barely changed for alcohol concentrations above 4% and 1.5% v/v, respectively. This behavior indicates that the micelle is mainly modified at lower alcohol concentrations by introduction of the molecule of 1-butanol and 1-pentanol into the micelle palisade. At high concentration of these alcohols, the molecules probably are dissolved in the micelle core.

It should be noted that the inserted oil in the ME droplets can affect micelle formation. According to Marsh *et al.* [13], the CMC of SDS for an SDS/octane/1-butanol ME was reached at 18 mM (0.5% w/w), instead of 8.3 mM (0.24% w/w) for aqueous medium. In all cases, the concentration of surfactant used for preparing a ME should be well above its CMC. This will produce enough micelles and ensure the system be dominated by stable oil droplets.

The effect of the concentration of SDS on solute retention and peak performance has been investigated by several authors. Some representative studied ranges are: 0.05–0.15 M (1.4–4.3% w/w) [15] (a range usual in MLC [9]), 1.75–5% w/w [4], 2.5–4.5% w/w [16], and 2.9–7.2% w/w [17]. The concentration finally selected was often relatively high inside the studied range, which is explained by the high lipophilicity of the analytes [18]. Thus, for example, for the 2.9–7.2% w/w range, the selected concentration for routine use was 5.8% w/w, since it provided good peak efficiency and highest resolution [17]. Although well above the CMC of SDS, the system could not solubilize the oil below 1.75% w/w, and thus, the ME could not be formed. Also, MELC was found unstable below 3% w/w SDS [13]. In another report, it was

commented that no ME was formed below 2.5% w/w SDS, and high back-pressure was generated above 4.5% w/w [16].

In the separation of isoquinoline alkaloids [19] (Fig. 2A) and phenolic compounds [20], 0.6 to 1.8% w/w SDS was the best range. The resolution decreased when the concentration of SDS in the mobile phase increased from 0.6 to 1.8% w/w. Decreasing the concentration down to 0.5% w/w dramatically increased the retention of the analytes, which was too large to be measured.

Authors found that, all over the investigated range, an increased concentration of SDS yielded shorter retention times for all analytes, or at least for some of them. This was explained by the distribution of solutes into the increased volume of the ME droplets (which act as pseudo-stationary phase), or the association of solutes at the droplets surface [21]. This made them travel with the speed of the mobile phase flowing towards the detector. These effects were more pronounced for lipophilic solutes, which have a high affinity for the oil droplets. The improved efficiency has been also attributed to the higher distribution of solutes into the ME droplets [22]. It seems that in the presence of oil and co-surfactant, an increased surfactant concentration increases the number of micelles in the mobile phase, but does not alter the layer of surfactant adsorbed on the stationary phase, and so its effect on solute retention. Therefore, the increased surfactant only affects the interaction of solutes with the ME droplets [13].

Some recommendations on the most appropriate concentration ranges for other less common surfactants can be found in the literature. Thus, the non-ionic Brij-35 was studied in the 0.5–2% w/w range. It was found that the retention was shorter with increased concentration in the 0.5–1% w/w range, which was explained by the modification of the stationary phase surface by the surfactant [23,24]. However, a further increase in the amount of Brij-35 had very small effect on the retention of analytes.

The non-ionic Genapol X-080 was investigated in the 0.4–2.0% v/v range for the separation of a group of flavonoids [25], and 0.5–2.5% v/v for phenylethanoid glycosides [26]. It was found that the retention decreased with increasing concentration of Genapol X-080 up to 1.2 and 1.5% v/v, respectively. Below 0.4–0.5% v/v, the ME was hardly formed or unstable. Above 2–2.5% v/v, there was very little effect on the retention of analytes.

2.2. Oil content

When the concentration of oil is zero, the mobile phase containing surfactant will have conventional micelles. The addition of oil to the micellar mobile phase decreases the retention, due to the increased mobile phase hydrophobicity. However, the observed effects upon changing the oil content in the ME mobile phase will depend on the nature of analytes. The effect will be stronger for lipophilic compounds. This can be explained by considering that an increase in oil leads to more oil droplets, where lipophilic compounds can partition. In contrast, hydrophilic compounds will have higher affinity towards the ME continuous phase, and therefore, they are not partitioned as fully into the oil droplets [13,24].

Different types of oil have been used to build the mobile phase in MELC (Fig. 3). Most often, alkanes (hexane, heptane and octane) and alcohols (mainly 1-pentanol and 1-octanol, with higher water-solubility than alkanes), of varying chain length, have been used. Other water-insoluble solvents used in analytical reports are cyclohexane, dichloromethane, ethyl acetate and diisopropyl ether. Some authors have indicated that changing the oil content had no significant impact on improving the separation performance. This is the case of a report using SDS/1-butanol/cyclohexane MEs, where the oil content was finally fixed at 0.9% w/w [27]. Ethyl acetate was investigated in the 0.5–1.0% w/w range, but only a slight decrease in retention of analytes was observed on increasing its concentration above 0.5% w/w [19,23,25].

However, other authors have reported significant effects on retention, selectivity and efficiency, and consequently, on the resolution of analytes by changing the oil content [28]. In general, the concentration range examined for oils is narrow. Thus, it was observed that increasing the diisopropyl ether content from 0.25% to 1% w/w, the efficiency and resolution increased, but these decreased slightly above 1% w/w [16,28]. On the other hand, for octane, the oil content was varied from 0 to 1.2% w/w, where no more oil could be solubilized by SDS. When the mobile phase contained 1% w/w or more oil, poor reproducibility was obtained, indicating that the system was unstable. An oil content of 0.8% was selected [13]. A more hydrophobic SDS/1-butanol/octane ME, capable of eluting insoluble compounds more quickly, was prepared by increasing the concentrations of surfactant and co-surfactant. This enabled raising the oil content from 0.8 to 2% w/w, so that the optimal oil:surfactant:co-surfactant ratio was kept, without compromising the ME stability [13].

One of the most recent advances in MELC is the use of ionic liquids as oils. The effect of different concentrations of the ionic liquid 1-hexyl-3-methylimidazolium hexafluorophosphate (HMIM·PF_6) was investigated in the 0.1–0.4% w/v range [20]. The optimal separation was achieved with a ME consisting of 1% w/v SDS, 3% w/v butanol, 0.2% w/v HMIM·PF_6, and 95.8% v/v water at pH 2.5, buffered with phosphoric acid and 10% v/v ammonia.

2.3. Concentration of co-surfactant

The co-surfactant has a very important role in the stability of MEs. It also influences the chromatographic behavior. By increasing the concentration of co-surfactant in the ME mobile phase, the proportion of organic phase in the aqueous component increases, and therefore, the solubilization effect. This reduces solute retention, especially for water-insoluble compounds [13,29,30]. Concomitantly, the elution speed for hydrophilic solutes is decreased [20].

According to some authors, the type and concentration of co-surfactant in MELC may have the biggest influence on the separation, but this again will depend on the analytes [21,31]. The most usual co-surfactants are 1-propanol and 1-butanol. Consequently, most studies on the effect of the concentration of co-surfactant on the chromatographic behavior in MELC correspond to these two alcohols.

An illustrative example of such studies refers to the determination of paracetamol in suppository formulations [32]. The addition of 1-propanol up to 5% v/v decreased only slightly the retention of this drug. However, the peak efficiency was significantly enhanced. No effect on retention and efficiency was found above 5% v/v 1-propanol. Also, it was found that above 3.8% v/v acetonitrile the ME became very cloudy and prolonged sonication was required to re-form it.

The concentration range for 1-propanol in a study to optimize the separation of four phenylethanoid glycosides was limited to 1–3% v/v [26]. The retention decreased with increasing concentration of 1-propanol from 1% to 2.5% v/v, but a further increase did not yield significant changes. Therefore, 2.5% v/v 1-propanol was used in subsequent experiments.

In other reports, the effect of co-surfactant concentration on the chromatographic behavior of a variety of compounds, using 1- and 2-propanol was investigated in the 5% to 15% v/v range. An increase in co-surfactant concentration resulted in decreased retention times [17,28,33]. Concentrations below 5% v/v yielded broad peaks and reduced sensitivity. Optimal performance (highest efficiency and best resolution) was obtained at relatively high concentration of 10–13% v/v. A higher concentration greatly increased the back-pressure, due to the high mobile phase viscosity.

In the optimization of the separation of several drugs, the concentration of 1-butanol was varied in the 6.6–16.5% v/v range [13]. Outside this range, the ME was unstable. The authors indicated that the ME system was able to accommodate increases in 1-butanol within the oil

droplets up to a concentration of 9% v/v, with little effect on the separation. Above this value, 1-butanol remained in the aqueous component, increasing the organic proportion in the mobile phase, which yielded faster elution for hydrophobic solutes. The chromatogram was thus compressed: the retention of the least retained compound remained constant, while for the other compounds it was decreased. This reduced the resolution.

In the implementation of a method for nifedipine in pharmaceutical formulations, an increase in the 5.6–8.6% v/v range for 1-butanol was observed to have no marked effect on the retention, whereas below 5.6% v/v an unstable ME system was obtained [16]. Concentrations above 8.6% v/v were not viable due to the increased column back-pressure. Experiments performed with different concentrations of 1-butanol showed that the retention of five isoquinoline alkaloids decreased noticeably along the 4–10% v/v range (Fig. 2B) [19]. However, with the highest assayed concentrations, overlapping of some peaks was visible. Concentrations below 8.0% v/v 1-butanol resulted in broad peaks and reduced sensitivity. Therefore, 8.0% v/v was selected as optimal, as it yielded the best separation with short analysis time. Other authors investigated a narrower 1-butanol range (0.5–3.5% v/v) to optimize the separation of a variety of drugs [23–25]. Changing the concentration of 1-butanol from 0.5% to 2.5% v/v, the retention times decreased. Since a further increase showed no marked effect on the retention, 2.5% v/v 1-butanol was selected in subsequent experiments.

3. Selection of pH

In aqueous-organic RPLC, retention of compounds is correlated with their polarity: more hydrophobic solutes will be longer retained. When compounds are ionized, they become more polar and retention decreases. Consequently, the mobile phase pH will strongly affect the retention of ionizable compounds. The same behavior is expected in MELC: retention of

ionizable compounds will be influenced by the mobile phase pH. However, the behavior is more complex with respect to conventional RPLC, since the interaction of the acid-base species with both ME droplets and modified stationary phase (especially for charged solutes interacting with charged surfactants) will shift the acid-base equilibria. One of the species will be stabilized changing the value of the dissociation constant (pK_a), as is also the case in MLC [34,35]. Therefore, the pH range where the retention of ionizable compounds is affected will be probably different in MELC and conventional RPLC. Meanwhile, for non-ionizable compounds, retention will be unaffected over the entire pH working range.

The effect of the mobile phase pH on retention, selectivity, and especially, resolution in MELC, has been studied by several authors. In an early report, the behavior of carboxylic acids (furosemide, bumetanide and naproxen), and basic compounds (atenolol, nadolol and timolol) was studied [21]. The pK_a values of carboxylic acids in aqueous medium are in the 3.5 to 5 range, and therefore, these compounds become increasingly ionized as the pH increases in acidic medium. As they become ionized, their retention decreases due to the weaker interaction with the stationary phase. The low affinity of carboxylate ions towards the ME droplets makes partitioning towards the pseudo-stationary phase neither possible. Similar behavior was found for naproxen (with $pK_a = 4.4$), which was examined above pH = 1.5 [13], and fosinoprilat [31], whose retention times were almost doubled by decreasing the mobile phase pH from 4.5 to 2.5. The pH was adjusted to 2.8 to attain good selectivity in a reasonable analysis time. In contrast, basic compounds are fully protonated in the 3.5 to 5 pH range, and thus their retention does not depend on the pH [21].

The behavior of hydrochlorothiazide and losartan potassium was studied at diverse pH values (Fig. 4) [22]. The two drugs differed in hydrophobicity (octanol/water partition coefficient, log $P_{o/w}$) and acidity constants (pK_a). For hydrochlorothiazide, log $P_{o/w} = -0.5$ and $pK_a = 7.9$, while for losartan, log $P_{o/w} = 3.3$ and $pK_a = 5.5$. Therefore, the retention time of losartan increased as the pH of the mobile phase was decreased below pH = 7. Meanwhile,

there was no significant effect on the retention of hydrochlorothiazide. It was also observed that at pH 7 the peak shape of losartan was poor and baseline separation could not be achieved between both compounds. A pH value of 5.0 was finally considered as optimal for the separation and detection of both analytes in a single run.

Dopamine receptor antagonist LE300 and its N-methyl metabolite were investigated using ME mobile phases in the 2.5 to 7.5 pH range [28]. Increasing the pH from 2.5 to 3.5 improved the efficiency and resolution. A value of 3.5 was selected since it yielded well-resolved peaks of high efficiency with reasonable analysis time. At pH > 3.5 the separation was poorer.

The effect on the MELC separation of six flavonoids in leaf extracts [25] and four phenylethanoid glycosides in rat plasma [26], was investigated in the 2 to 6 pH range, using phosphoric acid and increasing amounts of trimethylamine to adjust the pH. The retention time of the analytes decreased with increasing pH, indicating weak acidic character. A pH value of 6 was found optimal to get separation in sufficiently short analysis time. A similar recent study was carried out with eight phenolic compounds (also weak acids), in the 1.5 to 5.5 pH range using phosphoric acid and ammonium hydroxide to adjust the pH [20]. Strong co-elution was observed at pH 1.5–2.0 and above 5. Therefore, pH 2.5 was chosen as the most appropriate, since it provided good resolution within reasonable analysis time.

4. Effect of temperature

The effect of temperature on the chromatographic behavior of solutes in MELC was investigated by several authors. In an early study, Marsh *et al.* [13] injected a test mixture of parabens, oxibendazole and beclamethasone dipropionate at each of the following temperatures: 20, 30, 40, 50 and 60 ^{o}C. It was found that the separation was robust to temperature changes, since increasing the operating temperature had little effect on the retention times. However, peak-to-peak resolution between the last two peaks was enhanced. It was explained by the increase in solute mass transfer from stationary phase to bulk mobile

phase, droplet to stationary phase, and droplet to bulk mobile phase, at higher temperature. This resulted in a reduction in band broadening, especially for the most hydrophobic compounds. Average improvement of peak efficiency of 22% was observed for all peaks when raising the temperature from 40 to 60 ºC.

In a report with a test mixture of parabens, caffeine and paracetamol, the same results were found [36,37]. Raising the temperature from 20 to 60 ºC reduced the retention time by only 0.2 min for the early eluting compounds and by 1 min for those most retained. Instead, an important effect on the efficiency was found with improvements in the 20–70% range (Fig. 5). A similar chromatographic behavior was found for salbutamol and an internal standard from 20 to 50 ºC [24], and nifedipine from 25 to 45 ºC [16]. In another work, both peak retention and efficiency for paracetamol were affected by temperature, while peak asymmetry was unaffected [32].

5. Isocratic and gradient elution

One of the main strengths of the RPLC methods with mobile phases containing surfactant (MLC or MELC) lies in the capability of performing isocratic separations of mixtures of compounds exhibiting a relatively wide range of polarities, in convenient analysis times. In these chromatographic modes, the retention of the least retained compounds is often larger compared to conventional RPLC (which is interesting with regard to the direct injection of physiological samples). Meanwhile, the retention of the most lipophilic compounds is shorter (which has the advantage of the applicability of a single set of method factors to a wide range of analytes without needing gradient elution) [10,21]. Very short retention times could be obtained with isocratic elution by using a monolithic column at high flow rate and a ME prepared with 3.3% w/w SDS, 6.6% w/w 1-butanol, 0.8% w/w octane, and 0.05% trifluoroacetic acid (TFA) (Fig. 5) [36].

However, several authors have also suggested that gradient elution in both MLC and MELC could be useful to reduce analysis times for complex mixtures, with more fine control of retention than the isocratic methods. Gradient elution may offer benefits and enhance separation selectivity for hydrophilic and closely-eluting compounds. It was already proposed in MLC in the 90's for both the surfactant [38] and organic solvent [39]. Gradients of acetonitrile, methanol and 2-propanol, at constant micelle concentration, were shown to provide efficient separations. Among other applications, a gradient where both micelle and organic solvent were simultaneously increased, using SDS and 1-pentanol (where 1-pentanol played the role of oil) is worth to comment (Fig. 6) [40]. Initially, 0.05% v/v formic acid solution was pumped through the column and the amount of SDS/1-pentanol was increased. Successful separations of basic drugs (Fig. 6A), and neutral aromatics (Fig. 6B), were achieved in 14 and 4 min, respectively.

Other studies showed that MELC is suitable to carry out separations using gradient elution for a wide range of compounds. The gradients were usually formed with an aqueous starting eluent, which was mixed with increasing amounts of ME. A successful separation of 12 neutral aromatic compounds was obtained using a gradient formed by mixing from 5% v/v ME (SDS/1-butanol/octane/0.05% formic acid) up to 70% v/v ME at 15 min [40]. A gradient separation in less than 1 min was also reported for a mixture of paraben preservatives, using a monolithic column and a flow rate of 4 mL/min [36]. For these analyses, a linear gradient ramp was used starting with 5% ME/95% v/v TFA up to 100% ME. Also, an optimized MELC gradient method resolved, in 7 min, paracetamol and five related compounds potentially present in formulations [32]. The optimal gradient was started with 50% ME/50% v/v 0.05% TFA aqueous solution and ramped up to 100% ME in 8 min.

According to the authors, the methods gave superior peak efficiency and faster elution than MLC for the same test mixture, and superior selectivity than the MELC isocratic method. The MELC gradient methods were also superior to those in the conventional RPLC mode in terms

of analysis time, selectivity and efficiency. A remarkable characteristic was that there was no need for column re-equilibration between injections, since increasing the concentration of the ME droplets in the mobile phase does not change the structure and composition of the modified stationary phase, which is the case for hydro-organic gradients. The methods resulted in considerable time savings, compared to conventional RPLC. The ME gradient can be extended up to 100%, which is needed to elute highly hydrophobic compounds. This is not possible in conventional RPLC, due to column dehydration and difficulties in restarting the gradient.

In spite of the above benefits of MELC with gradient elution, there are also some negative comments in the literature that should be indicated. Thus, Bryant and Altria found that when a high proportion of water (at the start of the gradient) was tried to be mixed with an O/W ME, this was disrupted (demixed) in the pump, giving rise to an immiscible two phase suspension [40]. This made analysis impossible because the cloudy, unstable ME resulted in a very high and noisy UV signal. This problem was solved by increasing the ionic strength of the aqueous component by addition of NaCl. The presence of salt allowed a more easy formation of the ME at high water contents. Thus, the gradient was built by mixing 0.5 M NaCl solution (solvent A) with 3.3% w/w SDS, 6.6% w/w 1-butanol, 8% w/w octane and 10 mM sodium tetraborate (solvent B). The gradient started at 95% v/v of solvent A for 2 min, and decreased linearly to 25% v/v at 10 min. Under these conditions, the solutes were retained on the column until a sufficiently high ME composition was formed and each solute was eluted in turn.

Marsh *et al.* initially commented that they did not observe ME demixing in the course of the experiments, and the addition of NaCl was not required for a successful gradient [37]. However, later they found the ME/water problem, and concluded that the pumping and mixing properties of the chromatographic system affected the compatibility of the two mixed eluents.

388 According to McEvoy *et al.*, peak retention times and resolution were irreproducible in
389 MELC gradients. These authors indicated that reproducible separations can only be achieved
390 using isocratic MELC [41]. The irreproducible retention times in gradient MELC were
391 attributed to the nature of the adsorbed layer on the column packing and the possible
392 breakdown of the unstable ME produced during gradient elution. The authors affirmed that
393 reproducibility could be only achieved by allowing the column to equilibrate with the ME
394 mobile phase to get a constant adsorbed layer on the packing. When a concentration gradient
395 is employed, equilibration is not possible, since the nature of the adsorbed layer is constantly
396 changing during gradient runs. While MEs containing the cationic surfactant cetyl trimethyl
397 ammonium bromide (CTAB) remained stable during gradient runs, the problem of column
398 equilibration still remained owing to the changes in the adsorbed CTAB layer [41].
399 As O/W MEs are composed mainly of water, performing gradient elution by ramping up
400 the concentration of the aqueous component would not reverse the polarity of the eluent, and
401 may succeed in the separation of co-eluting compounds. Trying to exploit this idea, gradient
402 conditions were obtained by diluting an SDS ME from 100% to 0% v/v with its aqueous
403 component (0.05% TFA), in order to simulate a concentration ramp of the components during
404 gradient elution [41]. The diluted ME samples were visually examined to assess their
405 stability, based on the appearance of turbidity. Decreases in stability were also monitored
406 through the observation of possible increases in surface tension. At concentrations between
407 100% and 80% v/v ME, the system remained stable. When the ME was diluted to below 77%
408 v/v, it became turbid and the surface tension increased linearly up to the point where the CMC
409 of SDS was reached (approximately 8% v/v ME). Below the CMC, the surface tension of the
410 system rapidly approached that of water.
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6. Optimization strategies

As commented, most published work in MELC corresponds to isocratic elution. Accordingly, optimization strategies have been developed for this elution mode. Factors usually investigated include the type and concentration of surfactant and co-surfactant, the type of oil (less often its concentration), and pH in the mobile phase. As commented in previous sections, using adequate concentrations of surfactant and co-surfactant is essential to form a stable ME. If the oil:surfactant (or co-surfactant) ratio is incorrect, the ME will either fail or decompose into separate oil and water layers within a short period. Usually, the elution ability of the ME increases with increasing surfactant and co-surfactant concentrations. In general, all factors can alter the selectivity and efficiency.

The complexity of the composition of MEs will require many manipulations during method development, in order to achieve an acceptable separation for complex mixtures (i.e., sufficiently short analysis time and good resolution for the peaks of interest) [42]. In fact, one main problem of MELC is the many factors that should be optimized. This also makes robustness testing more demanding [43,44]. This is also the reason of the proposal from several authors of a selected ME as starting point, when developing a method for a new separation with no previous reports. For this purpose, a typical ME used in MEEKC was proposed in an early MELC work by El-Sherbiny *et al.*, consisting in 3.3% w/w SDS, 6.6% w/w 1-butanol, 0.8% w/w octane and 89.3% w/w aqueous solution of 0.05% TFA [21]. This ME, referred in MELC reports as standard ME (or standard conditions), is appropriate to separate mixtures containing compounds in a wide range of polarities. The same composition has been used in several reports [13,30,32,36,37,40,41]. Another suggested starting ME composition (2% w/w SDS, 10% w/w 2-propanol, 1% w/w 1-octanol and 0.3% w/w triethylamine in 0.02 M phosphoric acid) was inspired in a typical MLC mobile phase [21]. From these initial compositions, several modifications (one factor at a time) are usually performed to tune the analysis time and resolution in a controlled way.

Method development with trial and error approaches can be very time-consuming (especially when the number of factors is large). Also, these approaches often do not lead to the best experimental conditions. On the other hand, varying all factors affecting an MELC separation can generate a vast amount of data that further should be analyzed and interpreted. Therefore, it is desirable to get the best conditions by performing a limited number of experiments in a minimal time, using computer-assisted approaches. Several authors have been interested on the use of diverse Chemometrics tools to study the influence of each experimental factor in MELC, determine which are of primary importance, and find the optimal conditions for the selected factors. The approaches take benefit of the proper modeling of the main factors affecting the retention (concentrations of oil, surfactant and co-surfactant, to which pH should be added for ionizable compounds) [29,45–48].

Thus, experimental design techniques have been applied in several reports to optimize the composition of ME mobile phases. When several factors affect the separation, and these interact each other, their simultaneous optimization is needed. Using experimental designs, the number of involved experiments is significantly reduced, and the risk of missing non-linear relationships is minimized. However, some previous experiments should be performed to choose the suitable factors and determine the experimental domain. More often, 2^3 full factorial designs (where all input factors are set at two levels) were applied [27,29,48]. In a more complex study including the column type, a 2^4 full factorial design with four central point replications was developed [49].

New extreme values (star points) were also added to the full factorial designs, for each factor, to build a central composite design (CCD) [27,44,48,50]. This was used to create the matrix of experiments for mapping the chromatographic response surface to optimize the separation conditions. CCD produces a detailed quantitative model which can be used for the prediction of how a response relates to the values of several factors. This tool was applied combined with artificial neural networks in optimization and robustness testing [43].

In the optimization studies, only the resolution or both resolution and analysis time were taken into consideration. Multiple regression analysis of data was carried out to calculate the coefficients of quadratic polynomial equations, which correlate the response (such as the resolution) to the different factors (usually, the concentrations of oil, surfactant, and co-surfactant), as follows:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 \quad (1)$$

where y represents the estimated response, and x_1, x_2 and x_3 are three experimental factors. The model is mapped against two of these factors, while the third factor is held constant at the central value. As an example, Fig. 7 shows the response surfaces using different pairs of two factors in the separation of nine hydrophilic and hydrophobic components in *Salviae miltiorrhizae Radix et Rhizoma* (Danshen) [48]. The diagrams visualize the optimal conditions, but these can be finely obtained using software designed for optimization. Fig. 7 depicts also the chromatogram obtained at the optimal conditions. Addition to the ME of the cationic pair reagent CTAB below its CMC increased the retention and selectivity of acidic analytes. This surfactant couples with the anionic species to form a neutral complex that increases the hydrophobicity of these analytes.

In view of the complex ME composition and the requirement of simultaneous optimization of antagonistic objectives, some authors have employed multi-objective techniques using desirability functions (multi-criteria decision making tools) [16,48,51,52]. Genetic algorithms (GAs) were also applied to speed up the simultaneous optimization of the different factors involved in MELC separations [53]. GAs emulate the biological evolutionary theory and allows finding a global, true optimized condition among several possible local alternatives. In that work, the studied factors were the concentrations of oil, surfactant and co-surfactant, temperature and pH.

7. Preparation of ME mobile phases

The methodology used to prepare ME mobile phases is similar to that followed in MLC for hybrid micellar mobile phases, except for the fact that oil is added. First, an adequate amount of surfactant, most often SDS, is dissolved in a precisely measured volume of a solution of buffer, such as potassium dihydrogen phosphate. The pH is usually adjusted in the aqueous medium with NaOH or HCl [54]. Alternatively, the surfactant is first dissolved in water, and a buffer reagent is then added to fix the pH [13,55]. To prepare the ME, the co-surfactant (such as 1-butanol) and oil (such as heptane) are added to the surfactant solution, in this or the reversed order. After incorporating these and other additional reagents to the mixture, the solution should be stirred and sonicated in an ultrasonic bath during a few minutes (5 to 10 min). Diverse authors have also recommended sonication of the final mixture during 20 to 45 min [22,29,32,48,56]. Heating at 45 °C has also been suggested to obtain a stable ME [55]. Addition of each solvent should be carried out slowly while applying sonication to prevent disruption of the ME [32].

Mixing of oil, surfactant and co-surfactant before adding the aqueous component was also suggested [41]. The authors indicated that the ME was formed spontaneously upon addition of the aqueous phase to the other components, and remained unchanged during method development. In another procedure, ultrasonication was claimed unnecessary during the preparation of a ME containing SDS, 1-butanol, heptane and phosphate buffer [57]. According to the authors, standing for enough time with occasional shaking is favorable for the formation of a clear ME. The length of standing time depended on the composition of the ME and temperature.

In general, the successive steps of addition of the reagents, alternated with stirring in an ultrasonic bath, are important to get stable MEs. The mixture of oil, surfactant, co-surfactant, and water should be stored in a closed container, and left at room temperature for more than 12 h (or overnight), to make sure that a stable optically transparent ME is formed. If the

mixed solution is not clear after this period, the composition is unsuitable to form a stable ME.

In many reports, the authors claimed a sufficiently long period of good stability. Thus, at room temperature (around 25 °C), ME mobile phases have been described to be stable for at least 2 months [25,26], or even several months [31]. However, MEs formed with SDS, 1-propanol and diisopropyl ether, or SDS, 1-butanol, heptane and TFA have been indicated to be stable for only one week at room temperature [55,58]. The MEs could be made finally stable at 4 °C, yielding reproducible results as verified for at least one month.

8. Conclusions

ME mobile phases have been considered as environmentally friendly alternatives to the traditional solvents used in RPLC, owing to the low amount of organic solvent needed, and the reduced volume of mobile phase required because of the short analysis times. A great variety of different reagents suitable to form O/W MEs have been reported, together with a number of compositional choices that lead to the creation of a successful mobile phase. Selectivity can be altered by the type of selected oil, surfactant and co-surfactant.

A single set of experimental conditions has been shown to be applicable to the analysis of a wide range of different compounds. This recommendation helps in the selection of the best conditions, with time and cost savings compared to the need to optimize conventional RPLC and MLC conditions. If required, analysis time and resolution can be finely tuned by altering any of the several involved experimental factors. In this case, the number of operating parameters could make this task very laborious. It should be noted that retention times are often decreased by increasing the oil, surfactant and co-surfactant concentrations, but such changes can also affect negatively peak resolution. Changes in the mobile phase pH have similar effect on solute retention to that observed for MLC.

Gradient MELC has been recommended as a convenient method for drug stability studies. It offers the possibility of rapid determination of degradants and impurities in very hydrophobic formulations. However, there is some concern on the stability of MEs along the gradients and reproducibility of the results. Therefore, their implementation needs much deeper investigation.

Published work contains valuable information on how an MELC method should be implemented. However, the number of factors involved in this chromatographic mode can make such information rather misleading. This review was written based on a detailed study of the published work. The reported information has been compared and organized to facilitate analysts in the implementation of their MELC methods. This is an ideal technique to separate mixtures of compounds in a wide range of polarities, from hydrophilic to hydrophobic, being especially interesting for the analysis of highly water-insoluble samples.

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Figure captions

Figure 1. Schematic representation of an MELC system, showing the modified stationary phase and the oil droplets stabilized by surfactant and co-surfactant. White = polar head group of surfactant, black = polar head group of co-surfactant, and gray = solute (adapted from Xuan *et al.* [8]).

Figure 2. Effect of the concentration of surfactant (A) and co-surfactant (B) on the separation of five alkaloids from *R. coptidis* sample. (A) SDS concentration (%, w/v): (a) 0.6, (b) 1.0, (c) 1.4, and (d) 1.8; other experimental conditions were 0.8% w/v ethyl acetate, 8.0% w/v 1-butanol, 0.1% v/v acetic acid and 10% v/v acetonitrile. (B) 1-Butanol concentration: (a) 4.0, (b) 6.0, (c) 8.0, (d) 10.0; other experimental conditions were 0.8% w/v ethyl acetate, 1.0% w/v SDS, 0.1% v/v acetic acid and 10% v/v acetonitrile. Analytes: (1) epiberberine, (2) jatrorrhizine, (3) palmatine, (4) coptisine, and (5) berberine (reprinted from Ye *et al.* [19] with permission from Wiley).

Figure 3. Effect of the oil type (0.2% w/v oil) on the separation of phenolic compounds with UV detection: (A) ethyl acetate, (B) dichloromethane, and (C) octane. Other experimental conditions were 1% w/v SDS and 3% w/v 1-butanol at pH 2.5. Analytes: (1) sodium danshensu, (2) protocatechuic acid, (3) protocatechuic aldehyde, (4) caffeic acid, (5) lithospermic acid, (6) rosmarinic acid, (7) salvianolic acid B, and (8) salvianolic acid A (reprinted from Peng *et al.* [20] with permission from Elsevier).

Figure 4. Effect of pH on the chromatographic behaviour of: (1) Hydrochlorothiazide, and (2) losartan potassium (reprinted from Li *et al.* [22] with permission from Oxford Academic).

Figure 5. Isocratic separation of a mixture of paraben preservatives and paracetamol, using: (A) Hypersil BDS C18 column (150×4.6 mm i.d., 5 μm) at 1 mL/min, and (B) Chromolith RP-18e (100×4.6 mm i.d.) at 4 mL/min. Experimental conditions (w/v): 3.3% w/w SDS, 6.6% w/w 1-butanol, 0.8% w/w octane, and 0.05% TFA, 60 ºC, and UV detection at 215 nm. Analytes: (1) paracetamol, (2) methyl paraben, (3) ethyl paraben, (4) propyl paraben, and (5) butyl paraben (adapted from Altria *et al.* [36]).

Figure 6. Separation by gradient MELC with SDS and 1-pentanol (Reservoir A: water + 0.05% v/v formic acid; reservoir B: 33 g SDS and 100 g 1-pentanol dissolved in 1 litre of water with 0.05% v/v formic acid; detection at 240 nm): (A) Basic compounds (flow rate, 0.5 mL/min; gradient was 5% v/v B for 1 min, reaching 70% v/v B at 15 min). (B) Neutral aromatics (flow rate, 0.8 mL/min; gradient was 10% v/v B, reaching 100% at 3 min). Analytes: (1) norepinephrine, (2) isoproterenol, (3) atenonol, (4) pindolol, (5) lignocaine, (6) salmeterol, (7) labetalol, (8) bupivacaine, (9) phenacetin, (10) acetanilide, (11) acetophenone, (12) propiophenone, (13) butyrophenone, (14) valerophenone, (15) hexanophenone, and (16) heptanophenone (adapted from Bryant *et al.* [40]).

Figure 7. Three-dimensional resolution surface diagrams (left) for the separation of nine hydrophilic and hydrophobic components in Danshen with an Odyssil C18 column, considering the concentrations (w/w) of Brij-35, cyclohexane and 1-butanol, as factors. In each diagram, a factor was kept constant at the central level. Other experimental conditions were 85.6% phosphate buffer (pH 6.6), 8 mM CTAB, 30 ºC, 0.8 mL/min flow rate, and UV detection at 270 nm. Experimental chromatogram for the optimal conditions (right): 6.68% Brij-35, 0.84% cyclohexane and 6.92% 1-butanol. Analytes: (1) sodium danshensu, (2) caffeic acid, (3) protocatechualdehyde, (4) rosmarimic acid, (5) salvianolic acid C, (6) salvianolic acid B, (7) tanshinone I, (8) cryototanshinone, and (9) tanshinone II A (adapted from Huang *et al.* [48]).

Figure 1

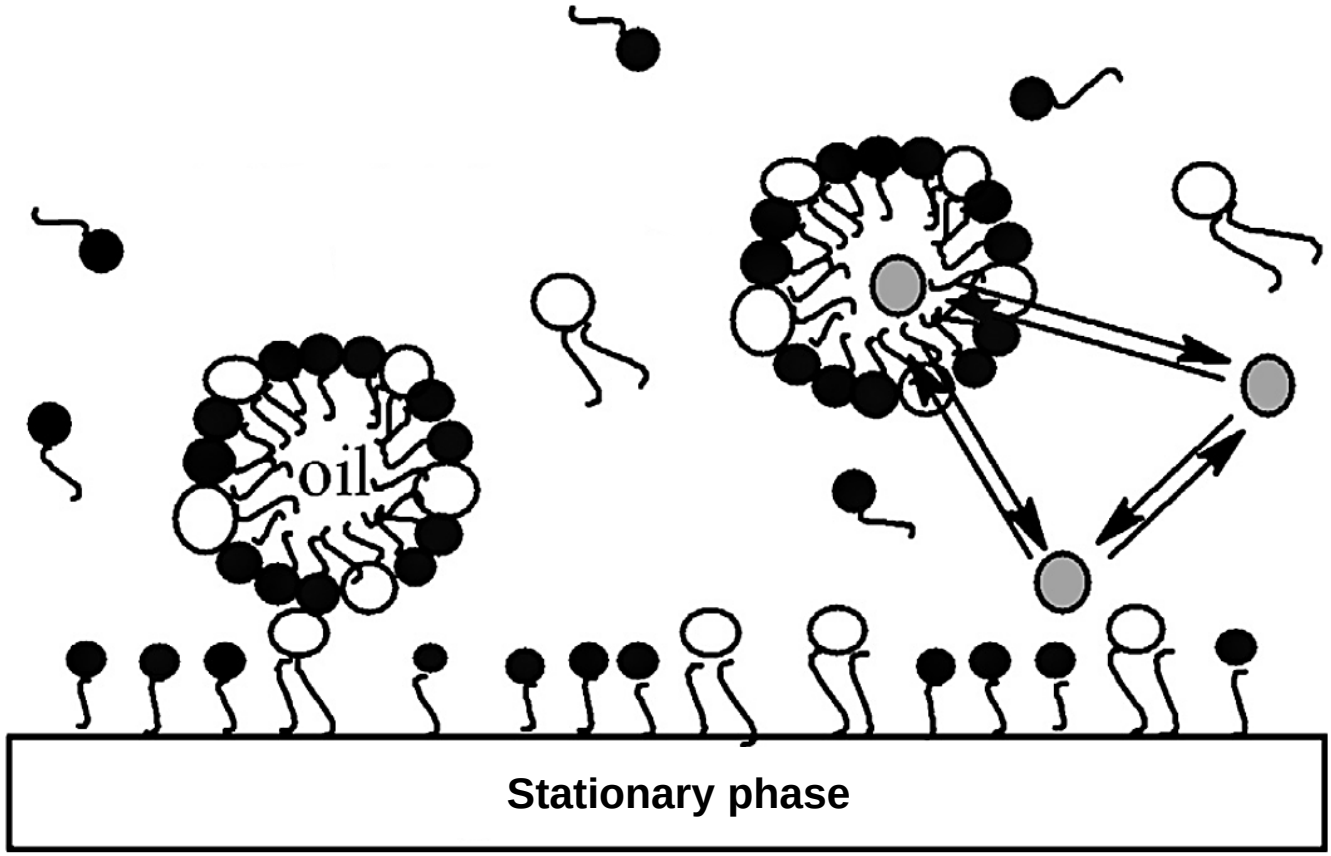

Figure 1
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Figure 2

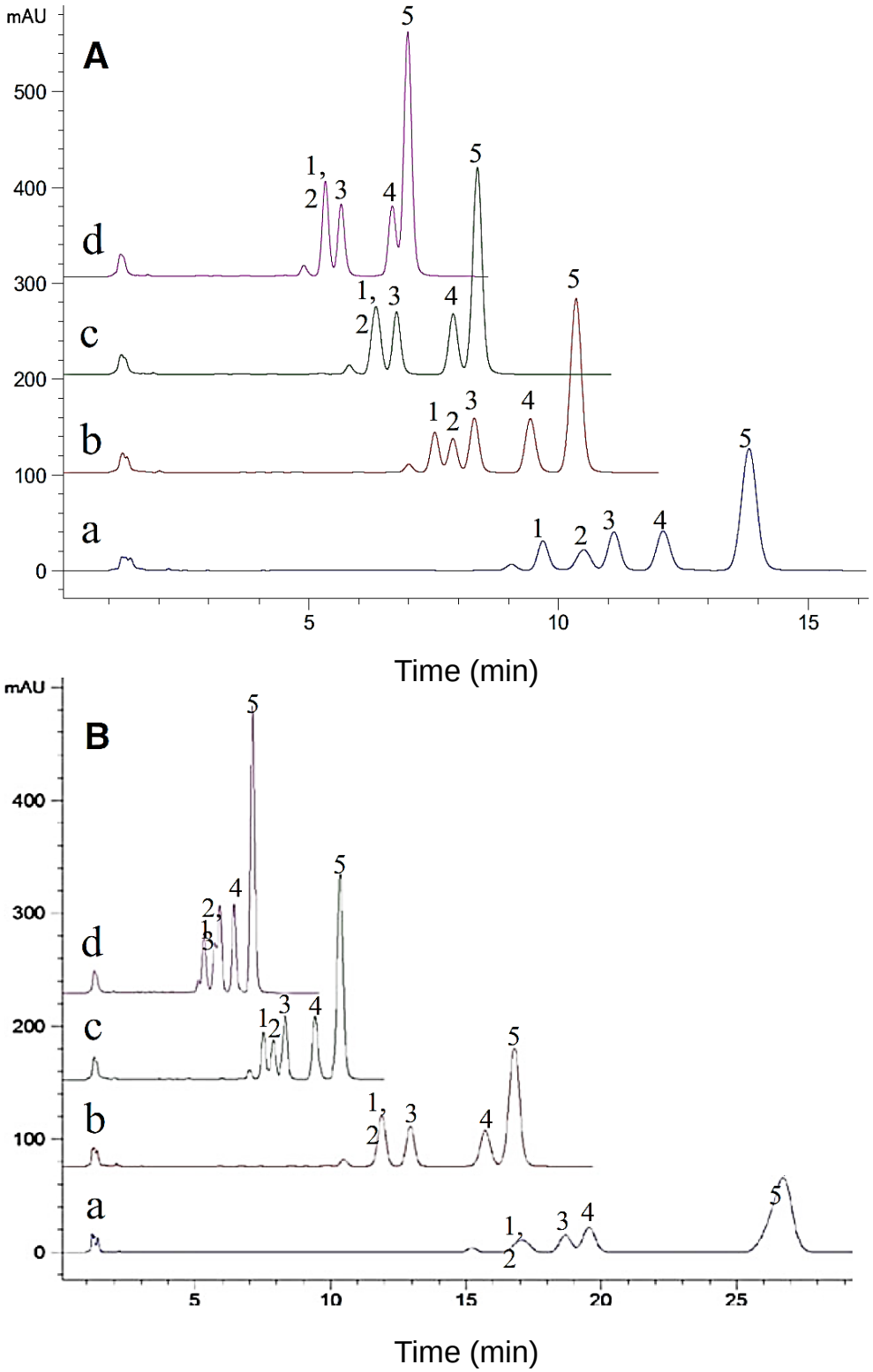

Figure 2
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Figure 3

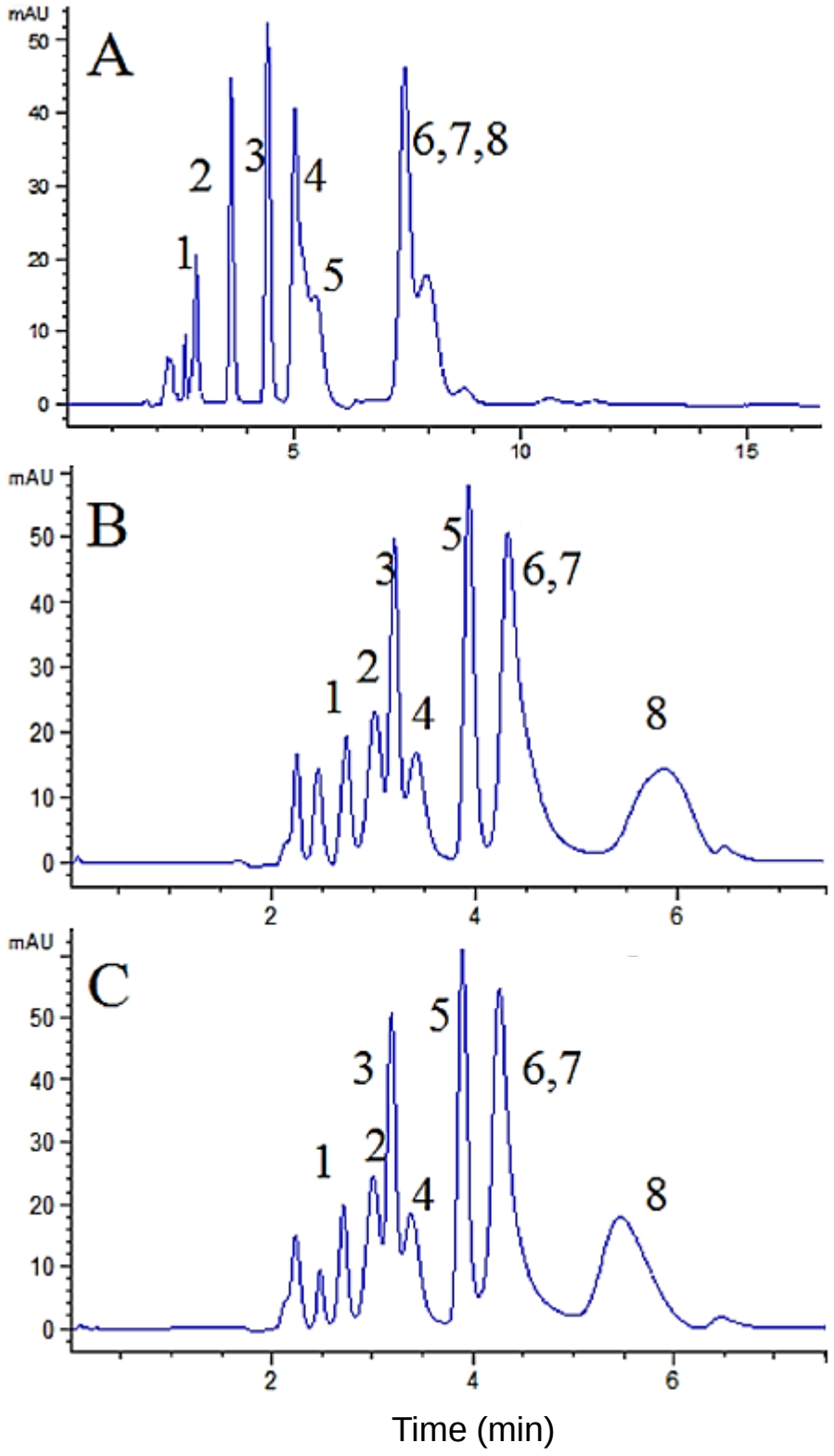

Figure 3
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Figure 4

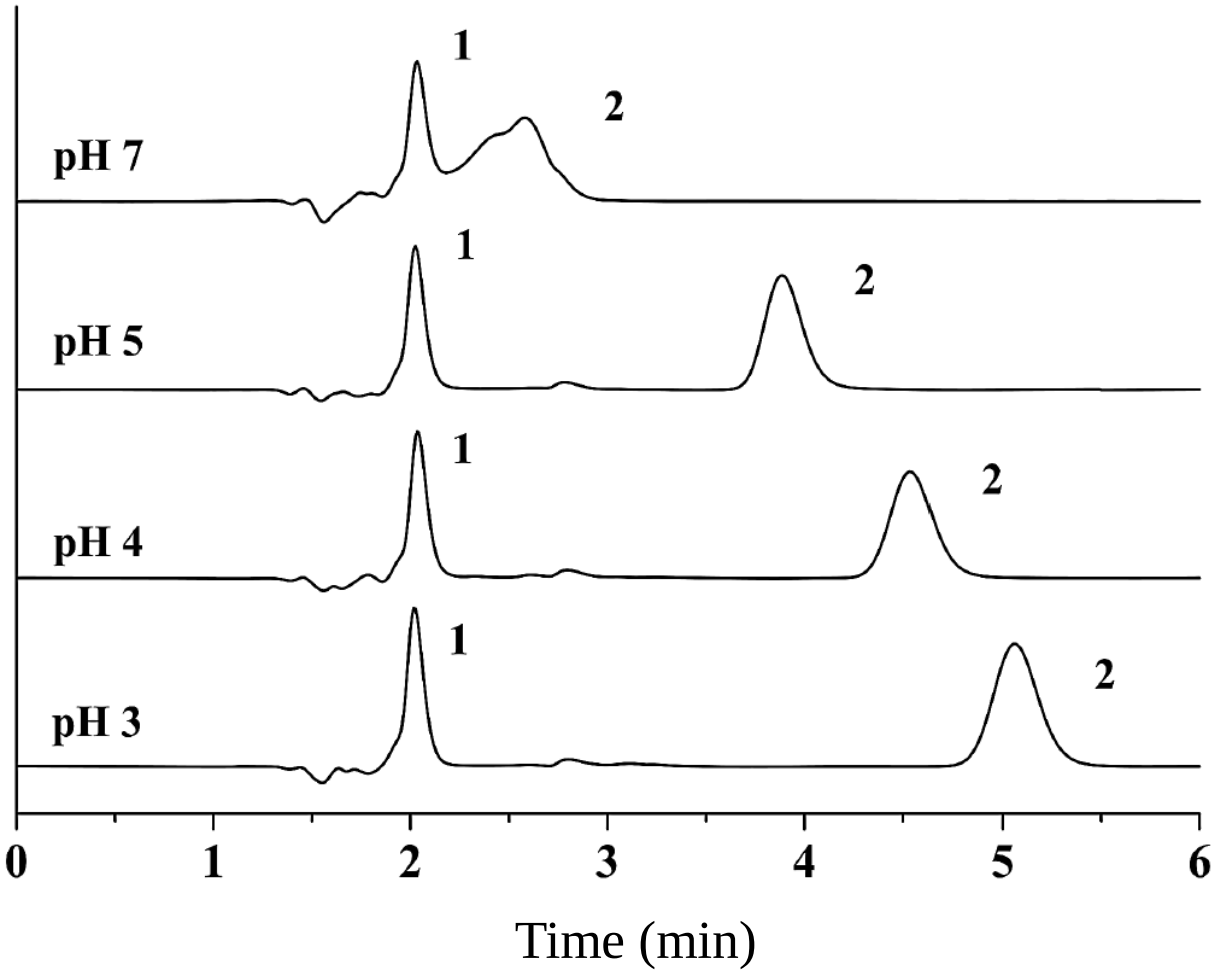

Figure 4
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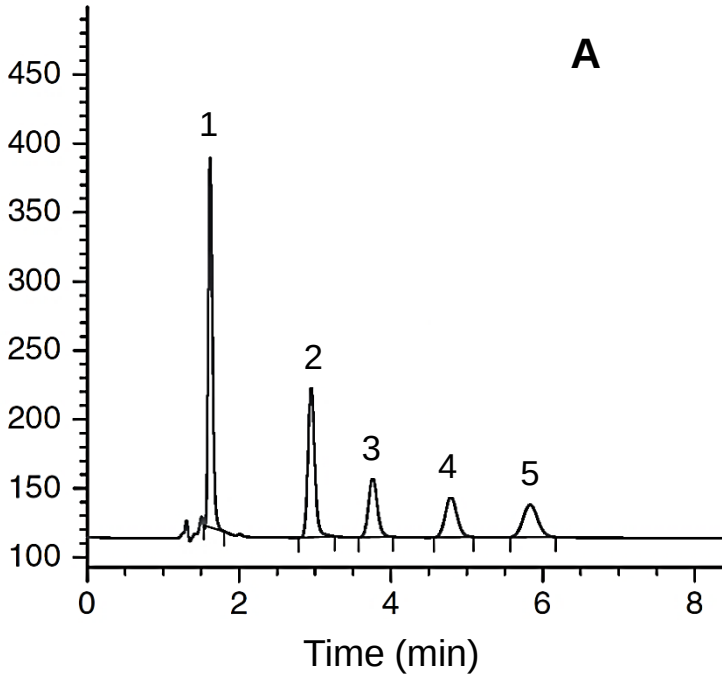
Figure 5



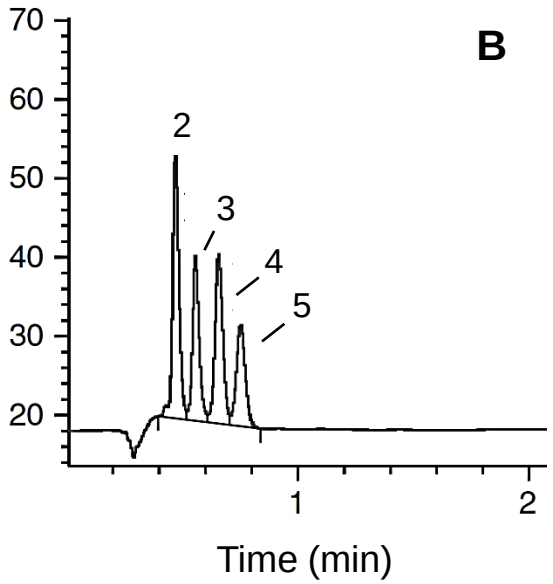


Figure 5
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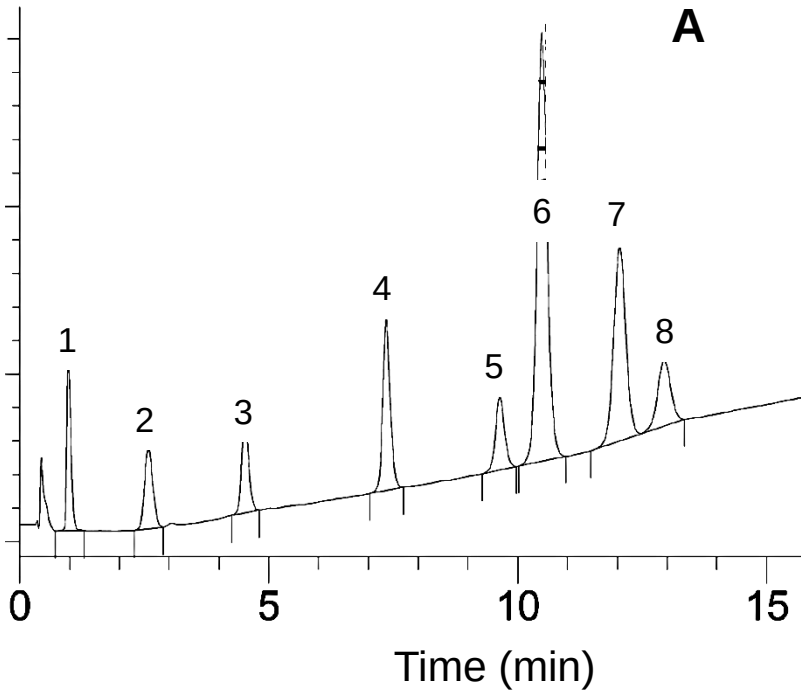
Figure 6



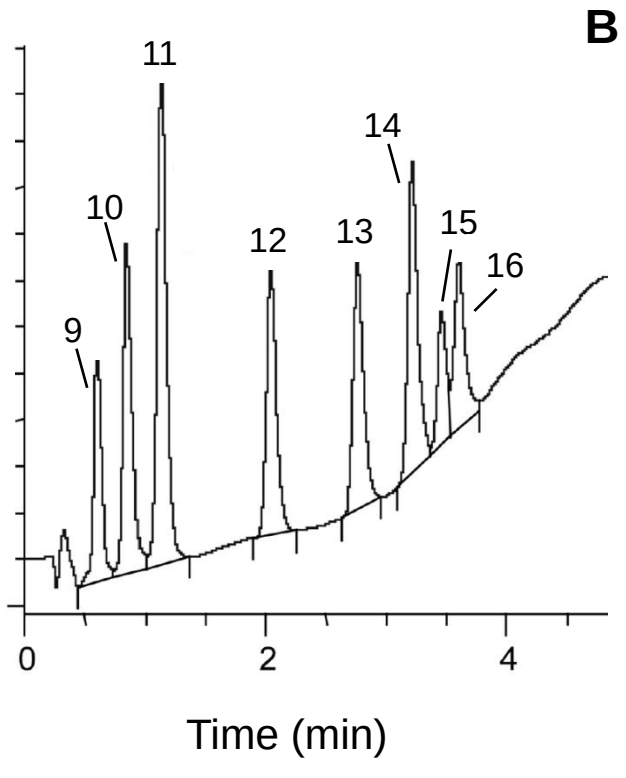


Figure 6
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Figure 7

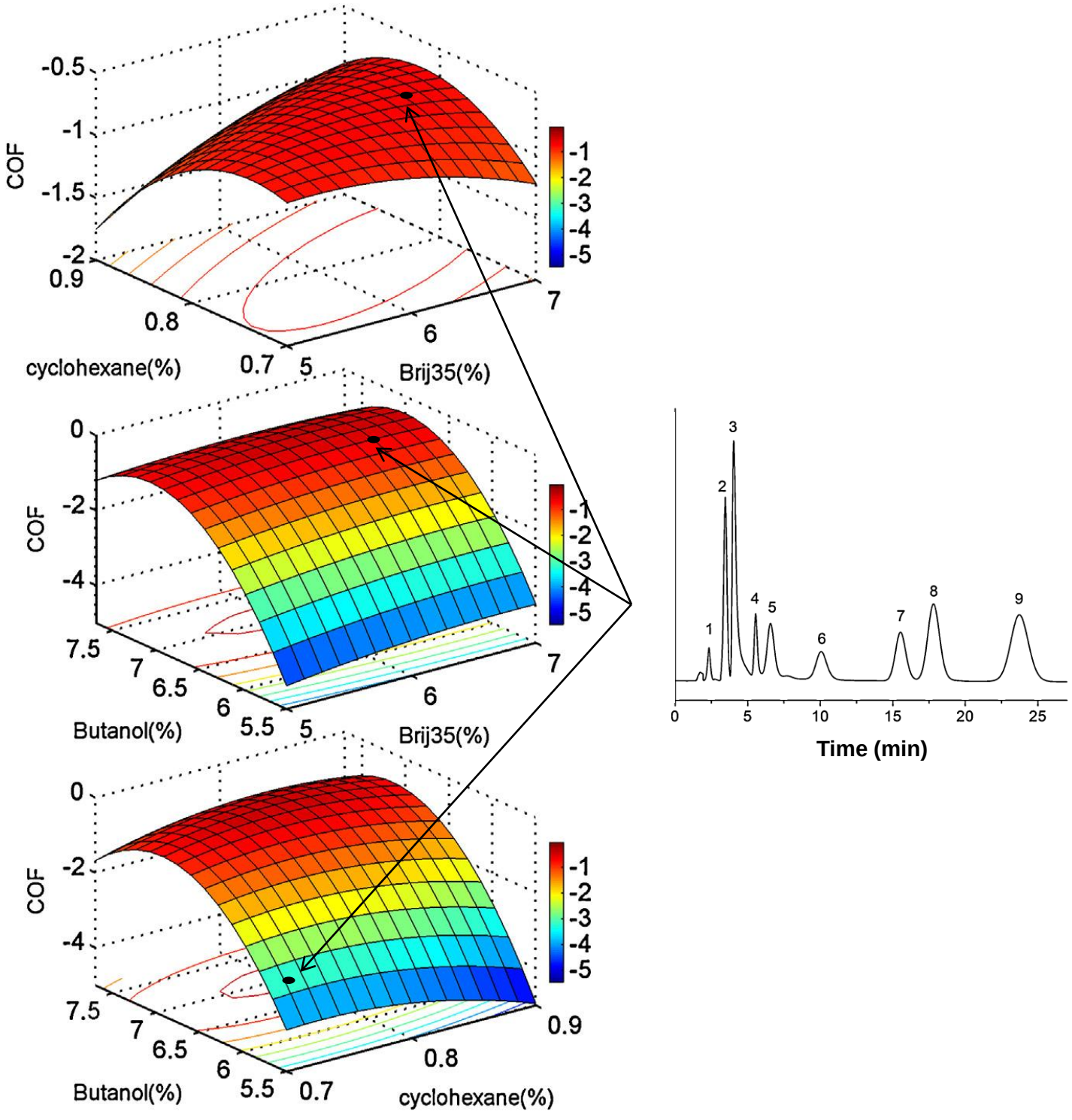

Figure 7
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