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Micellar liquid cromatography in bioanalytical chemistry

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Resum. En la cromatografia líquida micel·lar (MLC), la fase mòbil és composta per un surfactant i un alcohol. Els paràmetres que s'han d'optimitzar en la MLC són el tipus de columna, el pH, la naturalesa del surfactant i el modificador, i les seves concentracions. L'optimització es realitza utilitzant una estratègia interpretativa en la qual la millor fase mòbil se selecciona per a estudis de validació. Els mètodes optimitzats en MLC proporcionen separacions ràpides, eficaces, i permeten la determinació d'una gran varietat de substàncies en una matriu complexa. La MLC és una tècnica útil per a identificar medicaments en fluids biològics, que sovint es poden injectar directament al sistema cromatogràfic fins i tot en presència d'uns altres composts, incloent-hi proteïnes, sense cap altre pretractament que la filtració. En descriure les moltes aplicacions de la MLC, aquesta revisió inclou exemples agafats de la literatura, i també resultats recents de la mateixa recerca dels autors. Descriu l'aproximació teòrica, l'estratègia d'optimització i les aplicacions de la MLC en la determinació de grups diferents de substàncies de medicaments. Els compostos i les matrius en discussió són els següents: antidepressius en farmacèutics, agents de blocatge de canal de calci en sèrum, anserina i carnosina en mostres de carns, tiramina i triptamina en vins, i sulfonamides en llet.

Paraules clau: cromatografia líquida micel·lar (MLC) · estratègies de modelització i optimització en MLC · aplicacions de MLC en química bioanalítica

Abstract. In micellar liquid chromatography (MLC), the mobile phase is composed of a surfactant and an alcohol. The parameters that must be optimized in MLC are the type of column, the pH, the nature of the surfactant and modifier, and their concentrations. Optimization is performed using an interpretive strategy in which the best mobile phase is selected for validation studies. Optimized methods in MLC provide fast, efficient separations, thereby allowing the determination of a great variety of substances in a complex matrix. MLC is a useful technique for the identification of drugs in biological fluids, which often can be injected directly into the chromatographic system even in the presence of other compounds, including proteins, without any pretreatment other than filtration. In describing the many applications of MLC, this review draws upon examples taken from the literature as well as recent results of the authors' own research. It describes the theoretical approach, optimization strategy, and applications of MLC in the determination of different groups of drug substances. The compounds and matrices under discussion are: antidepressants in pharmaceuticals, calcium-channel blocking agents in serum, anserine and carnosine in meat samples, tyramine and tryptamine in wines, and sulfonamides in milk.

Keywords: micellar liquid chromatography (MLC) · modeling and optimization strategies in MLC · MLC applications in bioanalytical chemistry

Introduction

Micellar liquid chromatography (MLC) is a form of reversed-phase liquid chromatography (RPLC) in which the mobile phase is an aqueous micellar solution whose concentration is above the critical micellar concentration (cmc) [1,2]. The most widely used surfactant in MLC is anionic sodium dodecyl sulfate (SDS); others include neutral Brij-35 and cationic N-cetyltrimethylammonium chloride. MLC is a highly versatile technique that exploits the great variety of interactions between solutes, micelles, and the stationary phase. It is therefore appropriate for the analysis of a wide range of solute (hydrophilic and hydrophobic compounds), which can be separated in the same run.

A common practice in the most developed MLC procedures is to add a small amount of an organic solvent, usually a short-chain alcohol (methanol, propanol, butanol, or pentanol) or acetonitrile, to the mobile phase [2,3], resulting in hybrid mobile phases. The presence of the organic solvent increases the elution strength and improves the shape of the chromatographic peaks. For the resolution of complex mixtures, it is also important to properly adjust the pH of the mobile phase and to be aware of the acid-base side reactions of the solutes. Temperature and ionic strength should also be considered.

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This review begins by describing the optimization strategies used in MLC [1,17,18,22]. The development of a micellar analytical procedure for the determination of solutes in pharmaceuticals and biological fluids requires optimization of the column type, the type and concentration of the surfactant and organic modifier, pH, flow rate, and temperature. In addition, the optimization strategy can be sequential or interpretive. In a sequential strategy, a set of mobile phases is designed by taking into account the retention observed with previous eluents (the retention of the solutes is not known a priori). When the experiments are designed before the optimization process and used to fit a model that will allow the retention of each solute to be predicted, the strategy is called interpretative, and, of the two, it is more efficient and reliable. The need for an adequate experimental design becomes especially important when dealing with forms of liquid chromatography suitable for the simultaneous analysis of ionic and non-ionic compounds, such as MLC, in which several factors should be controlled. The strategy used to develop the method must provide answers to questions concerning the variables to be used as well as the set-up of the initial experiments that will yield an effective search of the appropriate variable space.

The second part of this review discusses the methodology used to develop a procedure for the control of several compounds in pharmaceutical, urine, serum, and food samples [9,13,14,19,21]. In RPLC, the determinations of a given family of substances are normally carried out either by isocratic elution with mobile phases of diverse composition or, if the polarity of the compounds is wide, by gradient elution. In the analysis of pharmaceuticals, however, the use of a unique mobile phase containing different drugs of diverse polarities is an attractive option.

Pharmaceutical formulations are commercially available in several forms (capsules, creams, drops, enemas, oily injections, ointments, pills, solutions, sprays, suspensions, syrups, and tablets). One of the main advantages of MLC in the resolution of mixtures of substances of different polarities is that the addition of just a small amount of an organic modifier to the mobile phase obviates the need for gradient elution [5,11,12].

Beyond the determination of drugs in biological fluids [2,3,6,7,13,18–20], MLC is a useful technique for the detection of compounds in food samples [14,21], which can be injected directly into the chromatographic system even in the presence of other compounds, including proteins. Moreover, as pointed out in this review, in MLC a single mobile phase is able to resolve several compounds of interest from complex mixtures, biological fluids (serum and urine), and food samples.

Modeling and optimization in MLC

Modeling of the retention behavior. The retention by SDS-modifier mobile phases can be modeled empirically (Eqs. 1–5) or mechanistically (Eqs. 6–9) [22] using the retention data of only five to eight mobile phases. Efficiency and asymmetry factors are also used to fit some of the equations.

In Equation 1, solute retention is linearly related to the mobile-phase variables within a selected portion of the optimization space and is fitted to a separate, logarithmic linear function.

$$\log k = c_0 + c_1[M] + c_2\varphi \quad (1)$$

Equation 2 establishes the hyperbolic relationship between retention and a fixed amount of organic solvent.

$$\frac{1}{k} = c_0 + c_1[M] \quad (2)$$

Equation 3 models the retention of a solute at varying concentrations of both the surfactant and the modifier.

$$\frac{1}{k} = c_0 + c_1[M] + c_2\varphi \quad (3)$$

Other empirical equations describe the retention behavior of any surfactant and organic solvent content [13,14,19] by considering the log *k* or *1/k* values related to the micellar concentration and the volume fraction of the organic modifier. Logarithmic models usually yield poorer results. Equation 4 is the simplest equation that provides good predictions for both polar and moderately polar compounds, such as amino acids, antidepressants, biogenic amines, β-blockers, sulfonamides, and diuretics. This equation yields linear plots of 1/k vs. φ at fixed concentrations of the surfactant.

$$\frac{1}{k} = c_0 + c_1[M] + c_2\varphi + c_3[M]\varphi \quad (4)$$

For highly hydrophobic compounds, Equation 4 plots are non-linear and an additional term is required, as shown in Equation 5.

$$\frac{1}{k} = c_0 + c_1[M] + c_2\varphi + c_3[M]\varphi + c_{11}\varphi^2 \quad (5)$$

The parameters in Eqs. 4 and 5 should be obtained by fitting the data in experimental designs with at least four and five mobile phases, respectively. However, at least one additional measurement should be made to check the accuracy of the fittings. The predictions obtained with Eqs. 1–3 are not accurate enough whereas the prediction errors of Eqs. 4 and 5 are usually <3% [5,11,21]. The parameters of the empirical MLC models are related to the physicochemical constants describing the interactions of the solutes with the three environments of micellar mobile phases: bulk water, micelles, and the stationary phase. A better understanding of the retention mechanism in micellar systems is provided by these models. Mechanistic models are based on Equation 2, which is the classical equation proposed for micellar mobile phases at a fixed volume fraction of organic modifier. Equation 2 can be re-written as Equation 6, which relates the retention of a solute to the concentration of monomers of the surfactant in the form of micelles.

$$k = \frac{K_{AS}}{1 + K_{AM}[M]} \quad (6)$$

For SDS-modifier micellar mobile phases, Equation 6 can be expressed as shown in Equation 7 [12], and Equation 5 as

shown in Equation 8. In the latter equation, retention may assume an excessive dependence on φ and produce high errors when an extrapolation is made in a region of high modifier concentration.

$$k = \frac{K_{AS}\dfrac{1}{1+K_{AD}\varphi}}{1+K_{AM}\dfrac{1+K_{MD}\varphi}{1+K_{AD}\varphi}[M]} \quad (7)$$

$$k = \frac{K_{AS}\dfrac{1}{1+K_{AD1}\varphi+K_{AD2}\varphi^2}}{1+K_{AM}\dfrac{1+K_{MD}\varphi}{1+K_{AD1}\varphi+K_{AD2}\varphi^2}[M]} \quad (8)$$

Equation 9 was proposed as an alternative model for highly hydrophobic solutes that takes into account the additional change in the concentration of solute associated with the stationary phase produced by the presence of modifier [12]. The constants K_{MD} and K_{AD} describe the displacement of the water-micelle equilibrium, while K_{SD} and K_{AD} represent modification of the water-stationary phase equilibrium.

$$k = \frac{K_{AS}\dfrac{1+K_{SD}\varphi}{1+K_{AD}\varphi}}{1+K_{AM}\dfrac{1+K_{MD}\varphi}{1+K_{AD}\varphi}[M]} \quad (9)$$

Equation 9 accurately describes the retention of solutes characterized by a wide range of polarities and eluted in SDS-modifier mobile phases [2].

Modeling of the peak profile. In MLC, an increase in the SDS concentration usually lowers the efficiency. Overall, MLC separations are characterized by a lower chromatographic efficiency than those achieved with aqueous-organic chromatography. Consequently, the equations describing the peak profiles are more complex. In MLC, the best peak-profile predictions are achieved using a Gaussian equation [1] in which the standard deviation depends polynomially on the distance to the peak time (Equation 10).

$$h(t) = H_0 e^{-0.5\left(\frac{t-t_R}{s_0+s_1(t-t_R)+s_2(t-t_R)^2+\ldots}\right)^2} \quad (10)$$

where $h(t)$ is the predicted signal at time t, H_0 the maximal peak height, t_R the retention time, and the coefficients s_i are related to the width and asymmetry of the chromatographic peak. For a given solute and mobile phase, t_R and s_i are ideally invariable, whereas H_0 depends on the concentration. This equation is also useful for graphical simulation of the chromatograms.

Modeling of peak resolution. In chromatography, the most widely extended optimization criteria to depict chromatographic performance are based on the calculation of an individual or elementary resolution measurement, r_i, for the least-resolved peak or peak pair, where p is the number of peaks or peak pairs and R is the global resolution (Equation 11).

$$R = MIN\,(r_i)\ 1 \le i \le p \quad (11)$$

These criteria are combined in MLC with the un-normalized product of Equation 11 and seem to be a better approach, although it can only be used with intrinsically normalized resolution measurements. This product varies from 0 (complete overlap between at least two peaks) to 1 (full resolution of each peak in the chromatogram). The global resolution function is combined with the elementary resolution as peak-to-valley measurements (Equation 12) and as overlapping fraction measurements (Equation 13) [11].

$$r_{i,i+1} = 1 - \frac{h_1}{h_2} \quad (12)$$

$$r_{i,i+1} = 1 - \frac{o_i'}{o_i} \quad (13)$$

where h_1 represents the height of the signal at a specific time to depict the valley location, h_2 is an interpolated height, measured at that time from the baseline to the line obtained by joining the maximums of the two neighboring peaks, o_i' is the area under a given peak overlapped by the chromatogram obtained from the remaining peaks, and o_i is the total area of the peak.

These strategies combined with computer analysis allow the simulation of chromatograms to predict peak retention and peak profile as accurately as possible [1]. The reliability of these simulations, assessed for different groups of compounds by comparing experimental and predicted chromatograms [3,5,6,11,20,21], is confirmed by the adequately low rate of errors. Thus, the mobile phase can be optimized and selected using this chemometric approach.

Determination of drugs in pharmaceutical formulations

Column selection. The most widely used columns in MLC are of the C18 type but, depending on the substances to be separated, other columns can be considered. The amount of surfactant adsorbed in the stationary phase has important implications regarding the retention behaviors of the solutes. Adsorption isotherms of SDS have shown that the amount of surfactant adsorbed by C8 columns is lower than that by C18 columns. Adsorption curves increase rapidly and reach a plateau at an SDS concentration higher than its cmc, which is 8.2 mM. For C18 columns, the adsorbed amount is constantly above the cmc, but for C8 columns the plateau is only reached at 300 mM SDS. The amount of SDS in the bonded phase is even lower for cyano-type columns and increases to at least 400 mM SDS with increases in the concentration of surfactant in the mobile phase [1].

pH selection. The pH is the first parameter to be considered in optimizing the composition of the mobile phase. For most columns, including C18-, C8-, and cyano-types, the working pH is restrained within a range of 3–9. Protonated substances interact with the negative charge of SDS, which increases their retention. For this reason, it is important to consider the protonation state of substances under study.

For anticonvulsants [16], benzodiazepines, and related compounds [17], a pH of 3 is appropriate based on the chemi-

cal properties of the substances. It is adequate when compounds are hydrophilic but can be positively charged, as is the case for β-blockers, carbamates, furosemide, and the vitamin B group [8,10]. The stability of the compounds at pH 3 is another factor to be considered. When the substances under study do not show an acid-base behavior or any other advantage at pH 3–9, pH 7 is preferred in order to extend column life.

Use of SDS and other surfactants. For pure micellar mobile phases, i.e., in the absence of any organic modifier, the chromatographic parameters, such as retention factors, asymmetries, and efficiencies, usually decrease as the surfactant concentration increases.

The most widely used surfactant in MLC is SDS ($C_{12}H_{25}OSO_3Na$), an anionic surfactant belonging to the alkyl sulfate group. An important property of surfactant solutions is that they form micelles at a concentration above the cmc. For SDS, with a cmc of 8.2 mM, the aggregation number (N_A) is about 70 monomers of surfactant per micelle at 25ºC.

Organic modifier selection. Most analytical procedures in MLC require the addition of a short-chain alcohol such as propanol, butanol, or pentanol, as its presence increases efficiencies. In SDS-modifier micellar mobile phases, the retention factor decreases as the modifier concentration increases, influencing the selectivity of the method. The proper selection of an alcohol is related to the polarity of the substances to be analyzed: propanol is useful for the most polar compounds, while pentanol improves the separation of hydrophobic substances.

It should be noted that very high efficiencies (3000–7000) have been obtained with hybrid mobile phases of SDS-butanol and SDS-pentanol. The upper reported efficiency values in MLC were generally below 4000. However, in the concentration ranges that were studied (50–150 mM for SDS, 3–6% (v/v) for butanol; 2–5% (v/v) for pentanol), the changes in retention produced by a change in the SDS concentration were higher than those produced by the modifier when butanol was used while pentanol displayed the opposite behavior.

Compared to RPLC methods employing aqueous-organic mobile phases, micellar mobile phases have the advantage of using small amounts of an organic modifier. Furthermore, propanol, butanol, and pentanol are retained in the micellar solution of SDS, thus reducing the risk of evaporation and increasing the stability of the micellar mobile phases. In addition, these alcohols are less toxic than methanol or acetonitrile, which are used in conventional RPLC.

Drug derivatization. The injection of sulfonamides into micellar mobile phases results in their appearance near the void volume, owing to their low hydrophobicity. Thus, to separate these substances using MLC, they must first be derivatized [21], e.g. using the Bratton-Marshall reagent, *N*-1-napthyl-ethylenediamine dihydrochloride (NED), as was the case in the study described in the following. To form the derivatives, sulfonamides are diazotized and coupled (Fig. 1). The azo dyes thus produced have a higher retention rate, thus enhancing the selectivity of the MLC method. Moreover, the resulting shift of the drug absorption bands to longer wavelengths upon derivatization facilitates their detection.

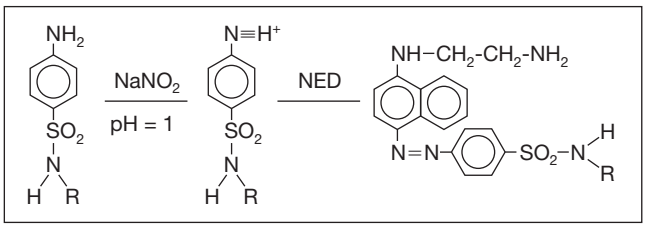

Fig. 1. Derivatization and coupling reactions for the UV determination of several compounds. (Reproduced with the permission of Elsevier [21]).

Method validation

One of the most critical factors in developing a chromatographic procedure to analyze pharmaceuticals is to ensure that the HPLC analytical test generates meaningful data. The International Conference on Harmonization Guidelines has introduced steps that must be taken in experimental work to define the appropriate validation characteristics; these may be considered simultaneously in the analytical procedure. They include, for instance, specificity, linearity, range, accuracy, and precision. Calibration graphs are usually constructed by at least a triplicate injection of five solutions of the drugs at increasing concentrations. Calibration curves are obtained by measuring the peak areas of each drug eluted with the optimum micellar mobile phase. Linear regression coefficients are usually $r > 0.999$. Limits of detection (LODs) are calculated using the *3s criterion*, which corresponds to a signal equal to three times the standard deviation of the background noise, i.e., the signal-to-noise ratio is equal to 3. LODs are usually below those required for the analysis of pharmaceuticals. Repeatability, or intra-assay precision, is determined using an average of ten measurements made on the same day. Intermediate precision is the average of ten repeatability measurements taken on 10 days over a 3-month period by different analysts, equipment, etc., and at three different drug concentrations in the range of the calibration graph.

Final validation of a MLC procedure aimed at determining the presence of pharmaceutical compounds includes a comparison of reference conventional RPLC with aqueous-organic mobile phases, to confirm the competitiveness of the latter. The performances of RPLC in the analysis of samples of two chromatographic modes should be compared, e.g., by injecting androgens and anabolic steroids [4] or sulfonamides [21] as drugs.

Two RPLC procedures using hydro-organic (11% acetonitrile) and micellar-organic (45 mM SDS-6% (v/v) acetonitrile) mobile phases were developed for the analysis of ten pharmaceutical formulations containing sulfacetamide, sulfadiazine, sulfaguanidine, sulfamethazine, sulfamethizole, sulfamethoxazole, or sulfathiazole. The composition of the selected mobile phases, formulated using algorithms, was determined using a classical empirical model for conventional RPLC and a mechanistic model for micellar RPLC. The two mobile phases were compared

with respect to determination of the drugs in pharmaceuticals the calibration curves, and the LODs, with the latter two being similar for the two cases. The determination of sulfaguanidine was the most problematic in the hydro-organic mode due to the low retention time of the compound. Nonetheless, the results showed that MLC is a competitive technique compared to conventional RPLC. Added advantages of MLC are the reduction in the amount of acetonitrile–in compliance with the increasing restrictions placed on the use of organic solvents in laboratories–and the use of cheaper and less toxic mobile phases.

Sample pretreatment in MLC

Conventional analytical aqueous-organic procedures can be replaced by those using micellar eluents in the control of pharmaceutical preparations, with good results. MLC offers important benefits over conventional RPLC; for example, a drug solution can be injected into the chromatographic system without the need for any treatment other than filtration, thus reducing sample preparation time. Although excipients are frequently not soluble in micelles, drugs are easily extracted when samples are treated with micellar solutions. The solubility of drugs is improved by the addition of a small amount of alcohol (methanol or ethanol) into the micellar media. The chromatograms of the pharmaceuticals show a peak in the void-volume region that corresponds to the excipients and to other compounds that are only retained to a slight extent (Fig. 2).

Sample preparation is very simple and depends on the pharmaceutical formulation: solids (capsules, tablets, pills, and powders), liquids (drops, solutions, suspensions, sprays, oily injections, syrups), ointments, and creams. In the case of solids, ten tablets or pills are weighed, ground to a fine powder, and homogenized. Several portions of this powder are selected, weighed and then dissolved with a suitable mobile phase. For capsules (typically, 10 units) are carefully emptied and cleaned and their contents weighed. With powders, the contents of 10 packets are mixed, homogenized, and the same procedure as used with tablets is then followed. For liquids, pretreatment is simpler and consists of homogenizing three aliquots, each of which is later mixed with a small amount of alcohol and diluted with a micellar solution. For more complex samples, such as ointments and creams, 1–2 g are mixed with the hybrid micellar solution, usually the mobile phase. If the sample contains high concentrations of fat-soluble excipients, an emulsion is formed in which case more hybrid micellar solution is added to further dilute the sample and to obtain a clear solution for analysis. The process is facilitated by the use of mechanical stirring and an ultrasonic bath. The recoveries obtained usually agree with the compositions declared by the manufacturers within the tolerance limits. Sample preparation time is shorter in MLC than in conventional procedures, which usually entail long and tedious extraction steps.

In drug determination of biological fluids such as serum and urine by MLC, the sample can be injected directly, without any pretreatment. SDS micelles tend to bind proteins competitively by releasing protein-bound drugs. Therefore, substances are free to partition into the stationary phase, and proteins are solubilized rather than precipitating into the column, eluting with, or shortly after, the solvent front.

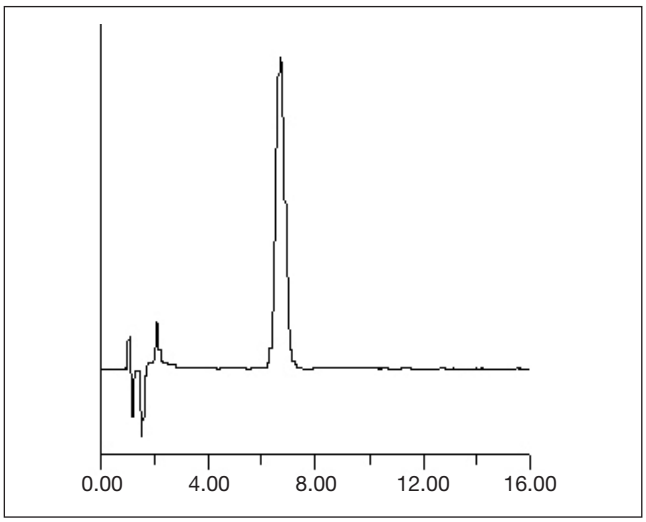

Fig. 2. Chromatogram showing the determination of a quinolone in a pharmaceutical.

Serum to be used for MLC analysis only needs to be centrifuged at speeds of 1400–3000 rpm for 10 min, which quickly separates serum from blood. Urine can be directly injected. Serum and urine are filtered before being introduced into the separation vial. When serum is injected into the chromatographic system, a wide band of proteins as well as several peaks corresponding to endogenous compounds appear at the beginning of the chromatogram, which could affect the detection of less retained substances (Fig. 3). The injection of a large number of serum or urine samples will shorten the life of the column or will require frequent regeneration of the stationary phase to avoid changes in the retention times due to matrix adsorption. To decrease the width of the protein band, serum and urine samples are diluted with a 0.9% NaCl solution.

Solid food samples, like meat, should first be ground finely using a mincer at 5000 rpm for 5 min and the homogenized sample then mixed with surfactant solution, followed by continuous shaking for 15 min. Afterwards, the homogenate is centrifuged at 3000 rpm for 5 min and the supernatant filtered and diluted in a SDS solution before its direct injection into the chromatographic system. Liquid food samples, such as milk,

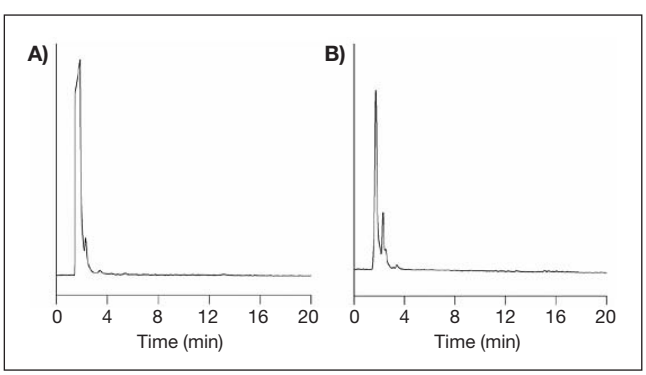

Fig. 3. Micellar liquid chromatograms in which the serum (**A**) and urine (**B**) blanks have been directly injected into the chromatograph.

can be directly injected into the chromatograph as described in the previous section for physiological samples.

There are some special cases, for example, sulfonamides [21], which prior to being injected are derivatized to improve selectivity, as discussed above. Sulfonamides are arylamines and contain a primary aromatic amine. This feature can be exploited in a diazotization-coupling reaction, as the resulting azo dyes can be detected and quantified in the visible band. Derivatization consists of three steps: (1) diazotization with sodium nitrite, (2) the elimination of excess sodium nitrite through reaction with sulfamic acid, and (3) coupling with the Bratton-Marshall reagent (NED). The use of a micellar SDS-containing medium offers several advantages, including alleviating the need to alter the pH, lowering the reaction time, and enhancing the molar absorption of the azo dyes.

MLC applications in bioanalytical chemistry

Antidepressant in pharmaceuticals and urine. Trazodone (TRZ) is a psychoactive compound that belongs to the group of second-generation non-tricyclic antidepressants used to relieve symptoms of depression. TRZ has been determined in pharmaceutical formulations and in urine samples [9]. Since the drug has a relatively low absorption in the UV region, spectrofluorimetric detection based on the native fluorescence of TRZ in SDS was implemented, with λ_{exc} = 325 nm and λ_{em} = 440 nm. In the following study, the optimized procedure consisted of a C18 column and a micellar mobile phase of SDS and 1-butanol. The mobile phase selected for use was 0.2 M SDS and 8% 1-butanol, with a pH fixed at 3 with phosphate buffer. The total analysis time was 10 min. Calibration in the TRZ pharmaceutical formulation was studied in the range of 0.3–5 μg/ml, yielding a good regression coefficient (r > 0.999) and good claim percentages (100.8–101%). The values agreed well with those declared by the manufacturer.

For the analysis of TRZ in urine, one main advantage is that samples do not require an extraction step. Calibration repeatability for the urine matrix was studied in at concentrations of 0.06–22.4 μg/ml, showing good linearity (r > 0.9991). The quantification limit was 9.5 ng/ml, thus ensuring the analysis of TRZ in biological fluids. The procedure showed good accuracy, repeatability, and selectivity. Repeatability and intermediate precision were tested for several concentrations of the drug, with high precision values (<2%). Good recoveries (93–110%) were obtained from spiked urine samples. No interferences from common additives frequently administered with TRZ or from endogenous compounds in urine samples were detected. The results were obtained with the optimized MLC method using samples collected from patients 6 h after TRZ administration (Deprax, 100 mg TRZ) (Fig. 4). In conclusion, this procedure is suitable for routine analyses of the drug.

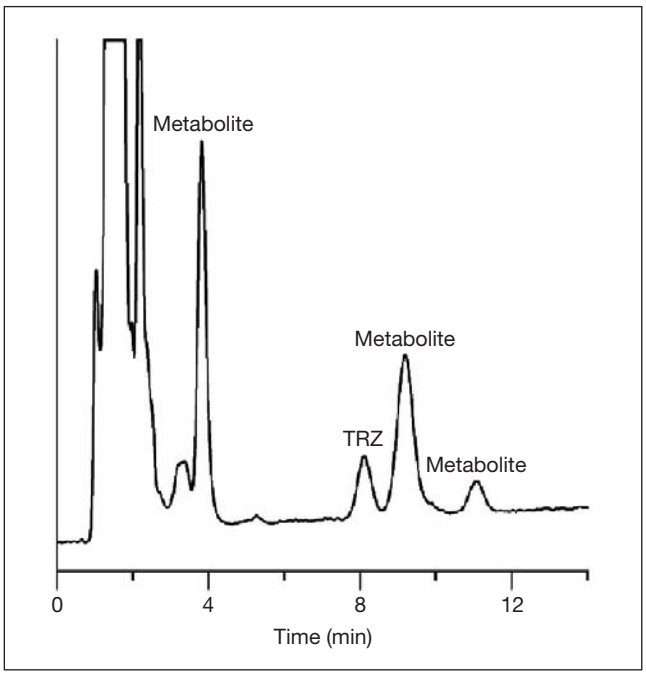

Fig. 4. Chromatogram of urine sample from a patient 6 h after the administration of the trazodone (TRZ) formulation Deprax, containing 100 mg of the active compound. (Reproduced with the permission of Elsevier [9]).

Calcium-channel-blocking agent in urine and serum. Verapamil [19] is determined in serum and urine samples by a sensitive and precise chromatographic procedure, without any pretreatment step, using fluorescence detection set at 230 nm (excitation) and 312 nm (emission). Verapamil is a phenylalkylamine derivate with calcium-channel-blocking activity. It is one of the most commonly prescribed drugs for the treatment of hypertension. Chemically, it is basic (log K = 9.1) with an equilibrium between two forms, one that is molecular and another that is positively charged. In the normal pH working range of C18 columns (3–7), the positively charged form of verapamil is predominant, with no changes in the retention behavior. In addition, verapamil is a highly hydrophobic compound (log $P_{o/w}$ = 4.6), such that the use of C18 columns and pure micellar mobile phases of SDS retention would be inappropriate. The mobile phase finally used to perform the analysis was 0.15 M SDS and 5% pentanol at pH 7. These conditions allowed the drug to be quantified in an adequate analysis time (12.5 min) without any interference by either the protein band or endogenous compounds.

Linearities were studied within a range of 100–2000 ng/ml in three different matrices (micellar solution, serum, and urine), and the regression coefficients were consistently r > 0.998. Intra- and inter-day precisions were also studied in the validation method. The LODs were 11.0, 18.5, and 20.2 ng/ml in micellar solution, serum, and urine, respectively. Recoveries in the biological matrices were 97–99%. Drug excretion in urine was studied in a volunteer receiving treatment for hypertension, and verapamil, as an unchanged drug, was separated from other metabolites (Fig. 5). The final quantity of verapamil eliminated was 5.04 mg, i.e., 2.8% of the dose taken. These results support the use of this procedure in the field of toxicology and clinical analysis.

Imidazole dipeptides in meat. The imidazole dipeptides anserine and carnosine [14] are well-studied natural antioxidants that are present in some types of meat. Interest in these com-

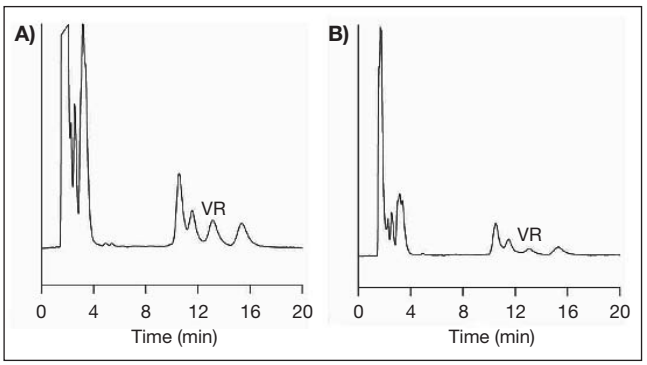

Fig. 5. Chromatograms of verapamil (VR) excreted in urine as unchanged drug 4 h after oral ingestion (**A**), and of residual verapamil (**B**). (Reproduced with the permission of Elsevier [8]).

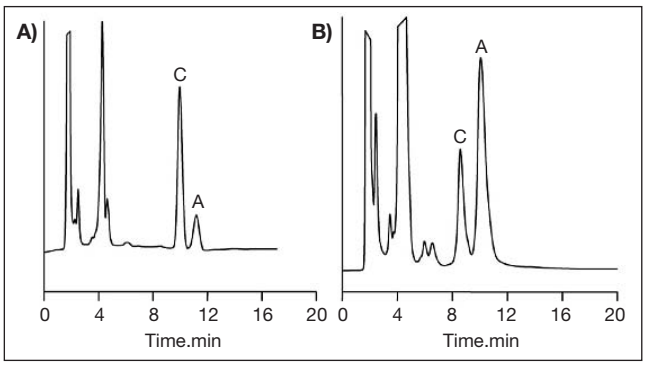

Fig. 6. Chromatograms of (**A**) a minced beef sample diluted 1:10 and (**B**) a chicken and turkey hamburger sample diluted 1:1. A, Anserine, C carnosine. (Reproduced with the permission of Elsevier [14]).

pounds in the area of food research derives from published studies of luncheon meats in which the meat species in processed meat products and the species incorporated into meat products were identified.

Three types of stationary phases (C18, phenyl, and amino columns) were examined at three different pH values (3, 5, and 7) in a procedure designed to determine anserine and carnosine in meat samples. Column performance was evaluated in terms of retention factor (*k*), efficiency (*N*), and peak asymmetry (*B/A*). The C18 column showed the highest retention at pH 3 (k = 42.3 for anserine and k = 53 for carnosine) and the worst resolution at pH 7. Efficiencies were N = 300–1000 and 200–600 for the phenyl and C18 columns, respectively. The quality of the chromatograms improved considerably when the amino column was used, with efficiencies and peak asymmetries of *N* from 1000 to 2600 and *B/A* from1.1 to 1.4. The two compounds were completely resolved without any interference from the protein band, thus confirming that the amino column is the most appropriate stationary phase for further study.

The retentions of anserine and carnosine decreased when the pH of the mobile phase increased. At pH 7, complete resolution and the lowest retention were achieved for both compounds. A pure MLC procedure was developed using a micellar mobile phase of 0.10 M SDS buffered at pH 7 and was followed by UV detection at 210 nm, with an analysis time of <12 min. The chromatographic parameters studied in this selected mobile phase were retention factor, efficiency, and asymmetry. The values obtained for carnosine and anserine were 8.9, 1939, 0.95 and 10.3, 1760, 1.1, respectively.

Several validation characteristics were tested. Calibration curves were constructed in the 1–50 µg/ml range on three different days (r > 0.998). The limits of detection (ng/ml) were 71 for anserine and 53 for carnosine. Repeatability and intermediate precision were evaluated at three different concentrations (1.5, 20, and 50 µg/ml) in meat matrices, with the residual standard deviations being <2.1%. In the evaluation of selectivity and recovery, recoveries values were around 100% without any interference from the protein band.

Meat samples (poultry, pork, and beef) were injected directly into the chromatographic system after extraction in a SDS solution followed by filtration (Fig. 6). The possibility of direct injection using MLC reduces costs and analysis time, in addition to decreasing error sources owing to minimized risks of losses and chemical changes in the analytes. Moreover, the selection of a pure mobile phase of SDS confers the procedure with a number of advantages over aqueous-organic solvent approaches, such as non-toxicity, non-flammability, biodegradability, and low cost. Its simplicity, therefore, makes it a good candidate for routine analyses in the area of food control and quality.

Biogenic amines in wines. Two biogenic amines [13], tryptamine (TryptA) and tyramine (TyrA), and their precursors, tryptophan (TryptO) and tyrosine (TyrO), were determined in wine by a liquid chromatographic procedure. Increased levels of biogenic amines in foods are of interest from several points of view, including the fact that at high concentrations they cause direct or indirect toxicity. Once these compounds are formed, they are difficult to destroy by pasteurization processes or cooking. Accordingly, it is important to control the production process. In fact, some countries have established regulations regarding intake limits or maximum overall limits of biogenic amines in various foods.

A hybrid micellar mobile phase of SDS and 1-propanol was used with a C18 column and electrochemical detection. A pH study in the range of 3–9 was performed, and pH 3 was finally selected based on the resolution and the analysis time. Oxidation potential at 0.6–0.9 V was also checked, with a maximum area obtained at 0.8 V. This potential was therefore selected for the subsequent analysis using a sequence pulsed amperometric detection waveform. The four compounds were resolved using a mobile phase of 0.15 M SDS and 5% 1-propanol, with an analysis time of 16 min.

Calibration curves were constructed using increasing concentrations in the 0.1–10 µg/ml range, with a linear response (r > 0.996). Repeatabilities and intermediate precision were evaluated at three different concentrations (1, 3, and 10 µg/ml) for each compound, yielding RSD values lower than 2.6 and 4.8%, respectively. LOD and quantification were obtained within the 10–40 and 33–135 ng/ml ranges, respectively. The applicability of the procedure was then tested in several types of wines and no matrix effect was observed (Fig. 7). These results show that direct sample introduction simplifies and greatly expedites these types of procedures, reducing their cost and improving their accuracy.

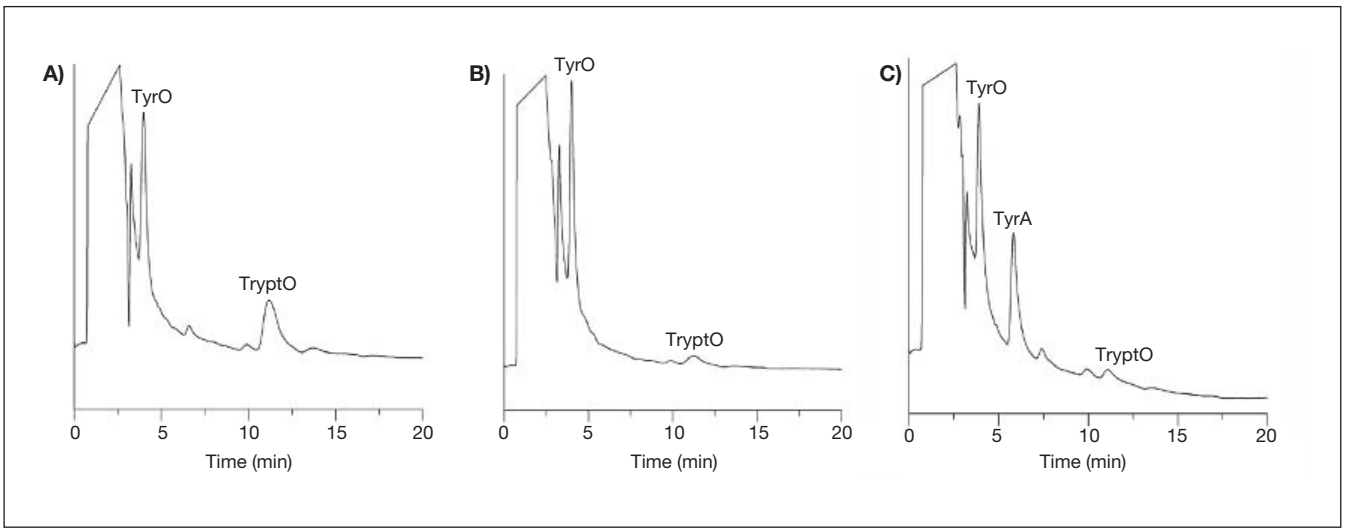

Fig. 7. Chromatograms of commercial wines: white (**A**), rosé (**B**), and red (**C**). (Reproduced with the permission of Elsevier [13].)

Sulfonamides in milk. Sulfonamides [21] are widely used for the prevention and control of a number of veterinary diseases, such as gastrointestinal and respiratory infections, as well as for growth-promoting purposes and prophylactically. Inappropriate abusive antibiotic-based treatments can result in undesired, persistent residues in animal tissues and in biofluids, including milk. Consequently, to prevent health problems the European Community has adopted a maximum sulfonamide residue level of 100 µg of total sulfonamides kg^{-1} in edible animal tissue, including milk. Therefore, a simple method to identify and determine six sulfonamides (sodium sulfacetamide, sulfamethizole, sulfaguanidine, sulfamerazine, sulfathiazole, and sulfamethoxazole) in milk by MLC was developed.

In the assay pre-column, diazotization-coupling was carried out as described above. Separation was achieved using 0.08M SDS and 8.5% 1-propanol at pH 7. The analysis times were <16 min and resulted in complete resolution (Fig. 8).

Good linearities ($r > 0.9999$) were obtained using calibration curves in the range of 0.2–20 µg/ml. The limits of detection and quantification were approximately 0.72–0.94 and 2.4–3.1 ng/ml, respectively. The detection limit was below the maximum residue limit established by the European Community. Intra- and inter-day precisions at three different drug concentrations (0.4, 6, and 17.5 µg/ml) were < 3.7%. Recoveries in spiked milk samples (0.4, 6, and 17.5 µg/ml) were 83–103%. The results demonstrate that this procedure is useful to screen and quantify sulfonamides in milk for routine analyses in the area of food control and quality.

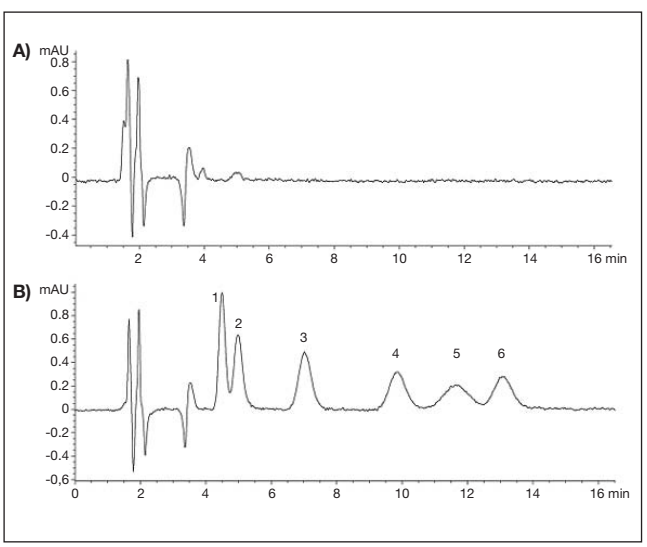

Fig. 8. Chromatograms obtained from milk samples: (**A**) blank milk treated with the diazotization-coupling reagents, with the azo-dye visualized at 490 nm; (**B**) milk spiked with 25 ng/ml of each sulfonamide. Sulfonamides and retention times (min) were: (1) sodium sulfacetamide, 4.5, (2) sulfamethiazole, 5.0, (3) sulfaguanidine, 7.0, (4) sulfamerazine, 9.9, (5) sulfathiazole, 11.6, and (6) sulfamethoxazole, 13.2. (Reproduced with the permission of Elsevier [21].)

Conclusions

This review of MLC described the optimization strategy used to select the best mobile phase for the resolution and determination of compounds in pharmaceutical preparations, serum, urine, and food samples. Compared to other eluents, micellar mobile phases are less flammable, cheaper, non-toxic, biodegradable, and can co-solubilize hydrophobic and hydrophilic analytes in complex matrices. In MLC, the elution of hydrophobic and hydrophilic analytes in the same run is possible without gradient elution. Furthermore, direct injection of physiological samples is feasible owing to the solubilization of proteins by the micelles and monomers of the surfactant. Additional features of this method are the possibilities of on-line analyte concentration and the ease with which selectivities can be changed by applying various separation additives. The pros and cons of MLC were outlined, and examples of the technique's application provided. Trends in MLC are quantitative structure–activity relationships (QSAR) studies, the mathematical description of mixtures of surfactants or modifiers, and the direct injection of bioanalytical samples.

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References

[1] Berthod A, García Alvarez-Coque MC (2000) Micellar Liquid Chromatography. Marcel-Dekker, New York

[2] Bose D, Durgbanshi A, Capella Peiró ME, Gil Agustí M, Esteve Romero J, Carda Broch S (2004) Micellar liquid chromatography determination of some biogenic amines with electrochemical detection. Journal of Pharmaceutical and Biomedical Analysis 36:357-363

[3] Bose D, Durgbanshi A, Carda Broch S, Gil Agustí M, Capella Peiró ME, Esteve Romero J (2005) Direct injection analysis of epinephrine, norepinephrine and their naturally occurring derivatives in serum by micellar liquid chromatography with electrochemical detection. Journal of Liquid Chromatography and Related Technologies 28:3265-3281

[4] Capella Peiró ME, Gil-Agustí M, Monferrer-Pons Ll, Esteve-Romero J (2002) Direct injection MLC method for the determination of corticosteroids in creams, ointments and other pharmaceuticals. Analytica Chimica Acta 454:125-135

[5] Capella-Peiró ME, Bose D, Gil-Agustí M, Esteve-Romero J (2004) Determination of Carbamate Pesticides Using Micellar Liquid Chromatography A. Indian Journal of Chemistry 42A:1417-1422

[6] Capella Peiró ME, Bose D, Durgbanshi A, Martinavarro Domínguez A, Gil Agustí M, Esteve Romero J, Carda Broch S (2005) Simultaneous determination of three opiates in serum by micellar liquid chromatography using direct injection. JAOAC 88:428-435

[7] Capella Peiró ME, Bose D, Gil Agustí M, Esteve Romero J, Carda Broch S (2005) Direct injection determination of benzoylecgonine, heroin, 6-monoacetyl-morphine and morphine in serum by micellar liquid chromatography. Journal of Chromatography A 1073:277-283

[8] Carda-Broch S, Rapado-Martínez I, Esteve-Romero J, García Alvarez-Coque MC (1999) Analysis of urine samples containing cardiovascular drugs by MLC with fluorimetric detection. Journal of Chromatography Science 37:93-102

[9] Carda Broch S, Gil Agustí MT, Monferrer Pons Ll, Esteve Romero J (2007) Determination of trazodone in urine and pharmaceuticals using micellar liquid chromatography with fluorescence detection. Journal of Chromatography A 1156:254-258

[10] Esteve-Romero J, Carda-Broch S, Gil-Agustí M, Capella-Peiró ME, Bose D (2005) Micellar liquid chromatography for the determination of drug materials in pharmaceutical preparations and biological samples. Trends in Analytical Chemistry 24:75-91

[11] Gil Agustí M, Carda Broch S, García Álvarez–Coque MC, Esteve Romero J (2000) Use of micellar mobile phases for the chromatographic determination of clorazepate, diazepam, and diltiazem in pharmaceuticals. Journal of Chromatographic Science 38:521-527

[12] Gil Agustí MT, Carda Broch S, Capella Peiró ME, Esteve Romero J (2006) Micellar liquid chromatographic determination of five antianginals in pharmaceuticals. Journal of Pharmaceutical and Biomedical Analysis 41:1235-1242

[13] Gil Agustí MT, Carda Broch S, Monferrer Pons Ll, Esteve Romero J (2007) Simultaneous determination of tyramine and tryptamine and their precursor amino acids by micellar liquid chromatography and pulsed amperometric detection in wines. Journal of Chromatography A 1156:288-295

[14] Gil Agustí M, Esteve Romero J, Carda Broch S (2008) Anserine and carnosine determination in meat samples by pure micellar liquid chromatography. Journal of Chromatography A 1189:444-450

[15] Khaledi MG (1997) Micelles as separation media in high-performance liquid chromatography and high-performance capillary electrophoresis: overview and perspective. Journal of Chromatography A 780:3-40

[16] Martinavarro A, Capella-Peiró ME, Gil-Agustí M, Marcos-Tomás JV, Esteve-Romero J (2002) Therapeutic drug monitoring of anticonvulsant drugs by micellar HPLC with direct injection of serum samples. Clinical Chemistry 48:1696-1702

[17] Martinavarro-Domínguez A, Marcos-Tomàs JV, Bose D, Durghbanshi A, Capella-Peiró ME, Gil-Agustí M, Esteve-Romero J (2003) Direct Injection of Serum Samples for Monitorization of Anticonvulsant Drugs. LabMedica International 20:1068-1760

[18] Martinavarro Domínguez A, Bose D, Durgbanshi A, Gil Agustí M, Capella Peiró ME, Carda Broch S, Esteve Romero J (2005) Monitoring bronchodilators with direct injection. Journal of Chromatography A 1073:309-315

[19] Rambla Alegre M, Gil Agustí MT, Capella Peiró ME, Carda Broch S, Esteve Romero JS (2006) Direct determination of verapamil in urine and serum samples by micellar liquid chromatography and fluorescence detection. Journal of Chromatography B 839:89-94

[20] Rambla Alegre M, Carda-Broch S, Esteve Romero J (2008) Development of a direct and validated method of amoxicillin in physiological fluids using Micelar Liquid Chromatography. Journal of Separation Science 31:2813-2819

[21] Raviolo MA, Rambla Alegre M, Clausell Tormos J, Capella Peiró ME, Carda Broch S, Esteve Romero J (2007) Determination of sulfonamides in milk after precolumn derivatisation by micellar liquid chromatography. Analytica Chimica Acta 593:152-156

[22] Torres-Lapasió JR, García Álvarez-Coque MC(2006) Levels in the interpretive optimisation of selectivity in high-performance liquid chromatography: A magical mystery tour. Journal of Chromatography A 1120:308-321

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