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Journal of Chromatography A, 1081 (2005) 77–86
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Analysis of ecdysteroids by micellar electrokinetic chromatography with on-line preconcentration

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Abstract

In order to enhance the UV detection sensitivity, an application study of an on-line preconcentration technique for micellar electrokinetic chromatographic (MEKC) was carried out. The simultaneous determination of four test ecdysteroids, 20-hydroxyecdysone, ajugasterone C, polypodine B and ponasterone A has been investigated by using the normal stacking mode in MEKC with UV detection. The effects of anionic surfactant composition and concentration, the applied voltage, the pH buffer, the kind and the amount of organic solvent and the injection time on the analyte resolution were evaluated. The optimised conditions for the separation involved the use of a 50 mM borate as the running buffer containing 50 mM of a mixture of sodium dodecyl sulphate (SDS) and sodium cholate (SC) in the ratio of 1:1 together with a concentration of 10% (v/v) of 2-PrOH at pH 9.0. Hydrodynamic injection of 12 s at 50 mbar and separation voltage of 20 kV at temperature of 20 °C were employed. These conditions allowed a repeatability separation within 21 min. Concentration detection limit for the neutral analytes studied improve about an order of magnitude. The method was also applied to the determination of ecdysteroids in a real sample.

Keywords: Capillary electrophoresis; MEKC; Ecdysteroids; Preconcentration; Stacking

1. Introduction

Ecdysteroids are steroids hormones that control moulting, metamorphosis and reproduction in insects. These steroids are present in all classes of arthropods and probably of other invertebrates too [1]. The identification of the same kind of compounds in several plant species (phytoecdysteroids, PEs) allowed their large availability and determined the development of numerous biological studies [2]. However, this subdivision must be regarded as non-strict because several ecdysteroid (e.g. ecdysone, 20-hydroxyecdysone (20E), ajugasterone C (ajuC)) are present in both insects and plants. The function of phytoecdysteroids in plants is still conjectural but they may be protective agents against phytophagous insects and soil nematodes.

A large number of papers dealing with ecdysteroids effects on mammals, including humans, are available in the literature. The pharmacological effects, that were especially ascribed to 20-hydroxyecdysone, were recently reviewed and critically analyzed by Lafont and Dinan [3]. The anabolic and growth-promoting effects of several PEs on mice, rats, pigs, quails and sheeps were reported long ago. Numerous preparations with declared roborant and tonic properties are based on the plant *Leuzea carthamoides* which possesses high levels of ecdysteroids, mainly 20E [4] and it is cultivated in Eastern Europe countries as a remedy in traditional medicine. Many other pharmacological effects have been also attributed to ecdysteroids. In fact, it was reported that these steroids hormones: (I) determine neuroprotective effect by reducing glutamate-induced cell death in cortex neurons of rat, (II) exhibit a hepato-protective action which was related to their effect on liver chromatin, (III) improve heart function, thence were recommended for the prevention of myocardial ischaemia and arrhythmia, (IV) have immunomodulatory effects, in humans by inducing the activation of lymphocytes and in mice by increasing, in the spleen, the concentration of antibody-forming cells, (V) have anti-inflammatory properties, in rats and mice, similar to cortisone acetate, (VI) possess antioxidative and anti-free radical properties in different

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doi:10.1016/j.chroma.2005.05.004

experimental models. Ecdysteroids have been also developed as effective inducers for gene switch control systems. Although no accurate toxicological studies have been performed for any ecdysteroids, there is evidence that these compounds have a very low acute toxicity in mammals [5]. All these pharmacological evidences, particularly the anabolic effect and the increase of physical performance, led to the development of a wide array of preparations containing PEs. About 150 different preparations for oral use can be found on the market. Nowadays, preparations based on purified PEs or containing crude/semipurified plant extracts are easily available by the Internet and most of them are proposed for use by bodybuilders.

Obviously, the remarkable scientific and commercial interest on this class of molecules requires the development of new methods of detection and quantification.

Analyses of ecdysteroids content on plant extracts by different techniques have been described. In 1994, a paper with 116 references [6] reviewed the chromatographic procedures for PEs with assessment of their strengths and limitations. In the recent literature few papers are available on the determination of PEs. In the last 10 years, traditional high-performance liquid chromatography (HPLC) [7,8] or specifically coupled with mass spectrometry (MS) [9] and NMR/IR [10] detections methods have been used. Recently, only three analytical methods using capillary electrophoresis (CE) have been reported. Two of them by micellar electrokinetic chromatography (MEKC) [11,12] and the third by capillary zone electrophoresis (CZE) [13].

CE has been established as a highly versatile and powerful technique in the separation sciences. It is an effective analytical tool for a large variety of analytes because it allows short analysis time together with high efficiency. The most widely used detector in CE is the UV–vis spectrophotometer and the major drawback is the low concentration sensitivity. Although CE can easily separate complex mixtures, this technique suffers from poor concentration sensitivity because small injection volumes needed to maintain high efficiency (2–10 μl) and the narrow optical pathlength is equal to the capillary diameter (25–100 μm).

Many different methods have been developed to improve the limits of detection (LOD) for CE. These involve the use of highly sensitive detection methods such as laser-induced fluorescence [14], electrochemical [15,16] and mass spectrometry [17] detection. Other investigations involve the installation of capillaries equipped with a bubble cell [18] or a Z-shaped flow cell [19]. An improvement of the LOD can be achieved using off-line preconcentration [20,21] or chemical derivatization [22]. However, all these methods are rather expensive or require time consuming procedures. Very interesting for improve detection sensitivity in CE, when using UV–vis detector, is the development of on-line sample concentration techniques.

A variety of on-line sample concentration techniques in CZE have been reported [23,24]. Four major on-line preconcentration methods have been reported in literature: stacking [25], sweeping [26], dynamic pH-junction [27] and transient isotachophoresis [28]. Each method relies on a various focusing mechanism based on different electrolyte properties between sample zone and background solution (BGS). These on-line concentration techniques can offer an increase of more than five orders of magnitude in concentration sensitivity.

The objective of the present study is to separate and concentrate on-capillary four test ecdysteroids: 20-hydroxyecdysone, ajugasterone C, polypodine B (polB) and ponasterone A (ponA) by using the MEKC with UV detection. The Fig. 1 represents the ecdysteroids investigate in our study that are generally recognised as four common active PEs. In order to improve concentration sensitivity of these neutral analytes, in this paper we report the development of a simple and rapid analytical method based on the on-line concentration technique for MEKC described by Quirino and Terabe [29], namely normal stacking mode (NSM).

The NSM is a simple on-line concentration method of sample. It is achieved by hydrodynamically injecting a sample dissolved in a low conductivity matrix. The mechanism of separation involves partitioning of analytes between the pseudostationary phase and the surrounding aqueous phase and electrokinetic phenomenon. The stacking effect for the analytes occurs at the interface between sample and buffer zones due to the different electric field strength.

The application of this technique under different experimental conditions has been performed. The method was then applied to the determination of PEs in a commercial extract of *Pfaffia paniculata*.

2. Experimental

2.1. Chemicals

20-Hydroxyecdysone, ajugasterone C, polypodine B and ponasterone A were obtained from SciTech (Praha, Czech Republic). Sodium dodecyl sulphate (SDS), sodium cholate (SC) and sodium taurodeoxy cholate (STC) were purchased from Aldrich (Milwaukee, WI, USA). H_3BO_3, NaOH, methanol (MeOH), ethanol (EtOH), buthanol (BuOH), propan-2-ol (2-PrOH) and acetonitrile (MeCN) were purchased from Carlo Erba Reagenti (Milan, Italy). All reagents were of analytical grade and used without purification. The water used for preparation of the solutions and running buffers was purified by a Milli-RX apparatus (Millipore, Milford, MA, USA).

2.2. Capillary electrophoresis apparatus and conditions

Electrophoretic analyses were carried out using a commercially available HP G1602A ^{3D}CE capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector (DAD) and an effectively air-cooling system. The data were collected on an

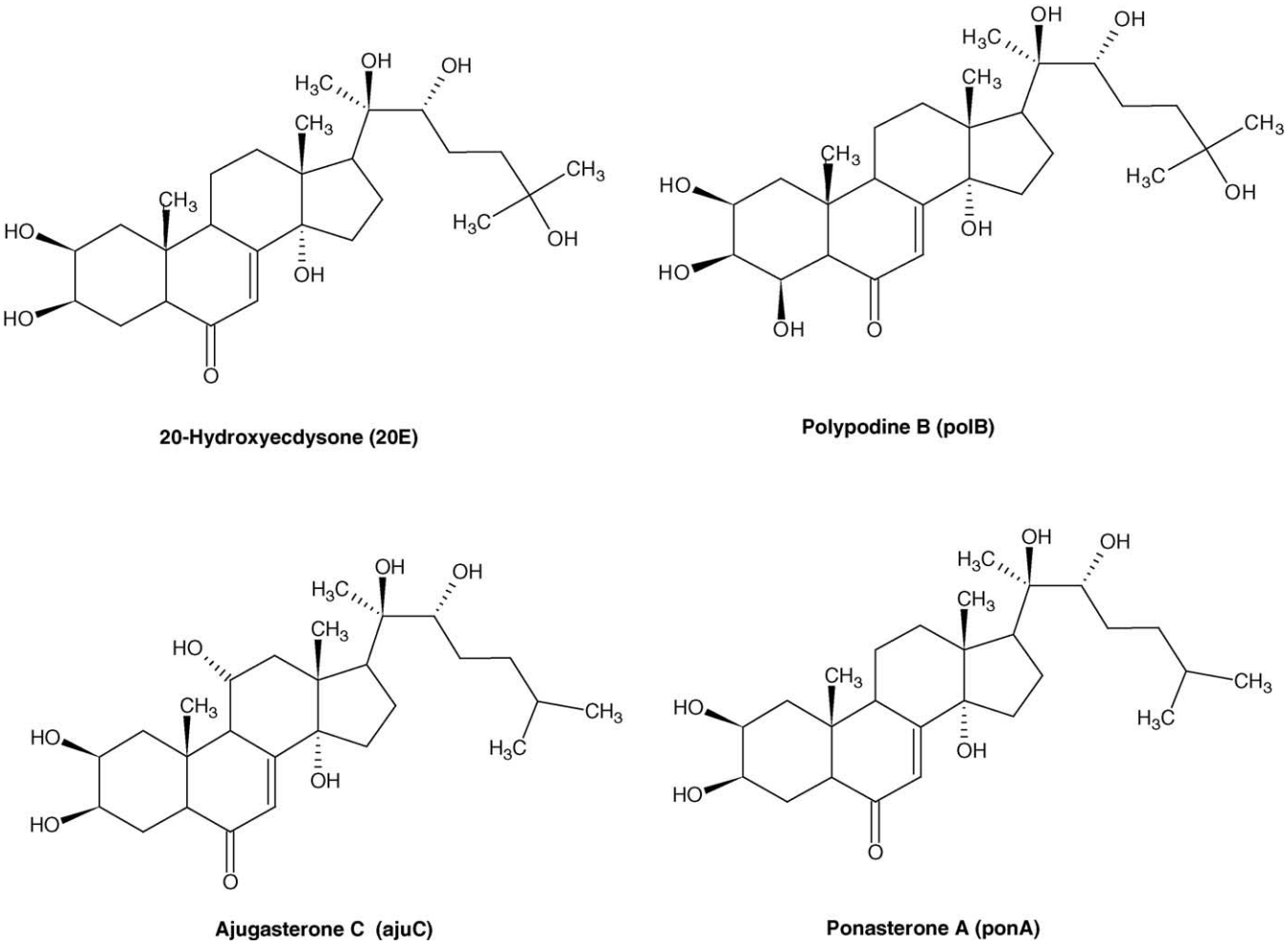

Fig. 1. Structures of the analyzed ecdysteroids.

Agilent ChemStation using a ^{3D}CE Software, Version 5.2. An untreated, fused-silica capillary (Agilent Technologies, Waldbronn, Germany) of total length 64.5 cm (effective length, 56 cm), 50 μm I.D. × 360 μm O.D. was used for separation. All separations were carried out at 20 °C with an optimised voltage maintained at 20 kV; hydrodynamic injections of 12 s were performed at 50 mbar and the detection wavelength was 254 nm.

Prior to first use the capillary was conditioned by flushing sequentially 1 M sodium hydroxide, 0.1 M sodium hydroxide, methanol, 0.1 M sodium hydroxide and finally water (20 min each). The capillary was equilibrated (10 min) at the beginning of the day with the running buffer. The repeatability of migration times was found to be strongly dependent on the rinsing procedure; the highest values of repeatability of the migration times were obtained by flushing the capillary between the runs as follows: 2 min with 0.1 M sodium hydroxide, 2 min with methanol, 2 min with water and 5 min with BGS. The BGS was prepared by weighting the suitable amounts of SDS–SC mixture, dissolving in aqueous solution of 50 mM sodium borate buffer (pH 9.0) and incorporating 2-PrOH (10%, v/v). Borate (0.1 M) stock buffer solution was prepared by dissolving appropriate amount of H_3BO_3 in purified water and adjusting the pH to 9.0 with 0.1 M NaOH. Borate buffer solutions (25–50–100 mM) were prepared by dilution of appropriate amount of stock solution. Buffer solutions and BGS were sonicated and filtered through a 0.45 μm filter (GyroDisc, Orange Scientific, Waterloo, Belgium) before use.

2.3. Method validation

The described method was validated. Linearity, detection limit, quantitation limit, accuracy and precision, were the validation characteristics that have been evaluated.

2.3.1. Linearity, detection and quantification limits

Stock solutions of ecdysteroids (1 mg/ml) were accurately weighted and dissolved in purified water solution containing the 55% (v/v) of EtOH and then diluted to give the working standard solutions. Standard solutions were daily prepared from stock solution by dilution with the same solvent mixture. All solutions were stored in a refrigerator set to maintain 4 °C. The linearity of the response was evaluated

Table 1
Repeatability, stacking efficiency (SE_{height}), linearity of response, limits of detection (LOD) and limit of quantification (LOQ) for the studied compounds (concentration: 70 μg/ml)[a] in NSM

Parameters	20E	polB	ponA	ajuC
Repeatability RSD ($n = 5$)				
Migration time (%)	0.73	0.72	0.69	0.70
Peak height (%)	1.1	1.8	1.6	2.0
Corrected peak area (%)	4.4	5.1	4.8	5.9
SE_{height}	11.7	12.0	10.1	12.0
Calibration line	$y = 0.12x + 11.08$	$y = 0.12x + 9.98$	$y = 0.13x + 19.06$	$y = 0.11x + 10.97$
Correlation coefficient	0.9958	0.9949	0.9950	0.9973
LOD (S/N = 3) (M)	7.2×10^{-6}	7.5×10^{-6}	7.0×10^{-6}	7.2×10^{-6}
LOQ (S/N = 10) (M)	21.0×10^{-6}	18.5×10^{-6}	23.5×10^{-6}	23.7×10^{-6}

[a] Experimental conditions: SDS–SC mixture, 1:1, 50 mM; pH 9.0 sodium borate buffer 50 mM and propan-2-ol, 10% (v/v). Fused-silica capillary (56 cm effective length) thermostated at 20 °C. Hydrodynamic injection (50 mbar × 12 s). UV detection at 254 nm. Voltage, 20 kV.

analysing standard solutions of all the studied ecdysteroids in the range 40–200 μg/ml. Triplicate injections were made for each standard solution and the calibration curves, constructed by means least-squares linear regression analyses, were obtained by plotting the ratios of the corrected peak areas (area/migration time) of each compound versus its concentrations. The correlation coefficients of the linear regression were greater than 0.99 (Table 1).

The baseline noise was calculated by Agilent ChemStation using a ^{3D}CE Software, Version 5.2 and signal-to-noise (S/N) values were determined dividing each peak height by the noise.

The values of detection limits (LOD) were determined from five runs, using progressively lower concentrations of analytes for a signal-to-noise ratio of 3. The quantification limits (LOQ) were determined from five runs and the S/N ratio was equivalent to 10. Table 1 lists the obtained values.

2.3.2. Accuracy and precision

The accuracy was evaluated by means of recovery studies. In order to determine the recoveries of 20E and polB, a measured volume of 10 ml of hydroalcoholic solution (water: 45% and ethanol: 55%) samples were spiked of each compound at three different concentration levels over the range of linearity. These spiked samples were extracted by the below described method. Aliquot of each of the three concentration levels were run in replicate of five. Extraction yield was calculated from the peak area of the analyte extracted from solution of plant root compared to the area obtained injecting standard solutions at the same concentration. The percent recoveries are listed in Table 2.

The precision of the method was assessed by testing the same set of spiked samples. The intra-day precision or repeatability was determined within the same day, in a single analysis on a single instrument. The results were expressed as relative standard deviation (RSD) for $n = 5$ and were summarized in Table 2.

2.4. Plant extract

A commercially available extract of *P. paniculata* (www.fitofarmaceutica.com) has been analyzed for its PEs content. Attempted analysis of commercial hydroalcoholic solution was unsuccessful because large and distorted peaks were obtained, probably due to the presence of interfering compounds. In order to remove polar and non-polar contaminants prior the analysis, a measured volume of 10 ml of hydroalcoholic solution (water: 45% and ethanol: 55%) of plant root was treated (two times × 5 ml) with $CHCl_3$. The hydroalcoholic solution was then treated with ethyl acetate (three times × 5 ml). The combined ethyl acetate solutions were evaporated to dryness under reduced pressure at a maximum temperature of 35 °C. The concentrate residue was redissolved in the same matrix of the test ecdysteroids (1 ml of water, 45% and ethanol, 55%; v/v), sonicated, filtered through a 0.45 μm filter and then was subjected to optimised MEKC analysis. In the analyzed sample, two PEs were found: 20E and polB (Fig. 5).

3. Results and discussion

3.1. Method development

The application of NSM in MEKC by using different kind of micelles (anionic, cationic, macromolecular) has been investigated for several analytes [30]. Up to now, there has been no report on simultaneous determination of ecdysteroids by using on-line preconcentration techniques in MEKC. Thus, the first aim of the present study was to provide a relatively

Table 2
Accuracy and precision for quantitation of 20E and polB in spiked hydroalcoholic solution ($n = 5$)

Compound	Amount added (μg/ml)	Mean amount detected (μg/ml)	RSD[a] (%)	Accuracy (recovery, %)
20E	40	34.40 ± 0.89	2.61	86.0
	70	56.98 ± 1.83	3.22	81.4
	100	90.20 ± 2.48	2.76	90.2
PolB	40	31.76 ± 1.05	3.31	79.4
	70	55.92 ± 1.72	3.08	79.8
	100	83.70 ± 1.66	1.99	83.7

[a] Relative standard deviation (RSD) = standard deviation of concentration detected/mean concentration detected × 100.

rapid and sensitive analytical method for the resolution and the determination of four principal ecdysteroids.

The close structural similarity of the four analytes (Fig. 1) requires a separation technique with high resolution and also a sensitive detection capability. In response to the sensitivity problem, different on-capillary focusing methods have been described in CE to preconcentrate analytes before the detection. Both charged and neutral compounds can be on-line preconcentrated. Sample stacking for ionic analytes was first introduced by Chien and Burgi [31], it relies on the movement of sample molecules along a boundary that separates regions of different electric field strengths. The sample zone and the BGS zone are the low- and the high-conductivity zones, respectively. When a voltage is applied the sample ions in the sample zone (high electric field region) move quickly and then slow down in the BGS zone (low electric field region). Consequently, the analytes are focused at the boundary of the two zones. It should be noted that by using electrophoretic effects only charged molecules can be preconcentrated. Since neutral analytes have no electrophoretic mobility, the application of this technique of preconcentration in MEKC requires the use of ionic surfactant micelles. Very interesting are the preconcentration techniques sample stacking and sweeping in MEKC those can be used individually or in combination. Recently, on-line sample concentration techniques in MEKC have been reviewed [30]. NSM in MEKC was first introduced by using sodium dodecyl sulphate in the sample matrix with a concentration greater than the critical micelle concentration (CMC) [32]. In the development of our method, the process of stacking has been performed by preparing the sample in water/EtOH (a matrix having a conductance lower than that the BGS), without the surfactant used in BGS and by injecting sample solution for a much longer time compared to usual hydrodynamic injection. All separations were performed on a length of 64.5 cm (effective length, 56 cm), and narrow bore (50 μm I.D.) capillary, at temperature of 20 °C and a voltage of 20 kV to reduce analysis time and limit the generation of excessive operating current (20–25 μA). The elution order of analytes was 20E (12.98 min), polB (13.55 min), ajuC (14.73 min) and ponA (19.81 min). Fig. 2 shows a typical electropherogram obtained by analysing a mixture of pure and commercially available standard 20E, polB, ajuC and ponA ecdysteroids. The run time was 21 min. Finally, a comparison between the applied on-line preconcentration technique and the usual analysis without on-capillary preconcentration was made.

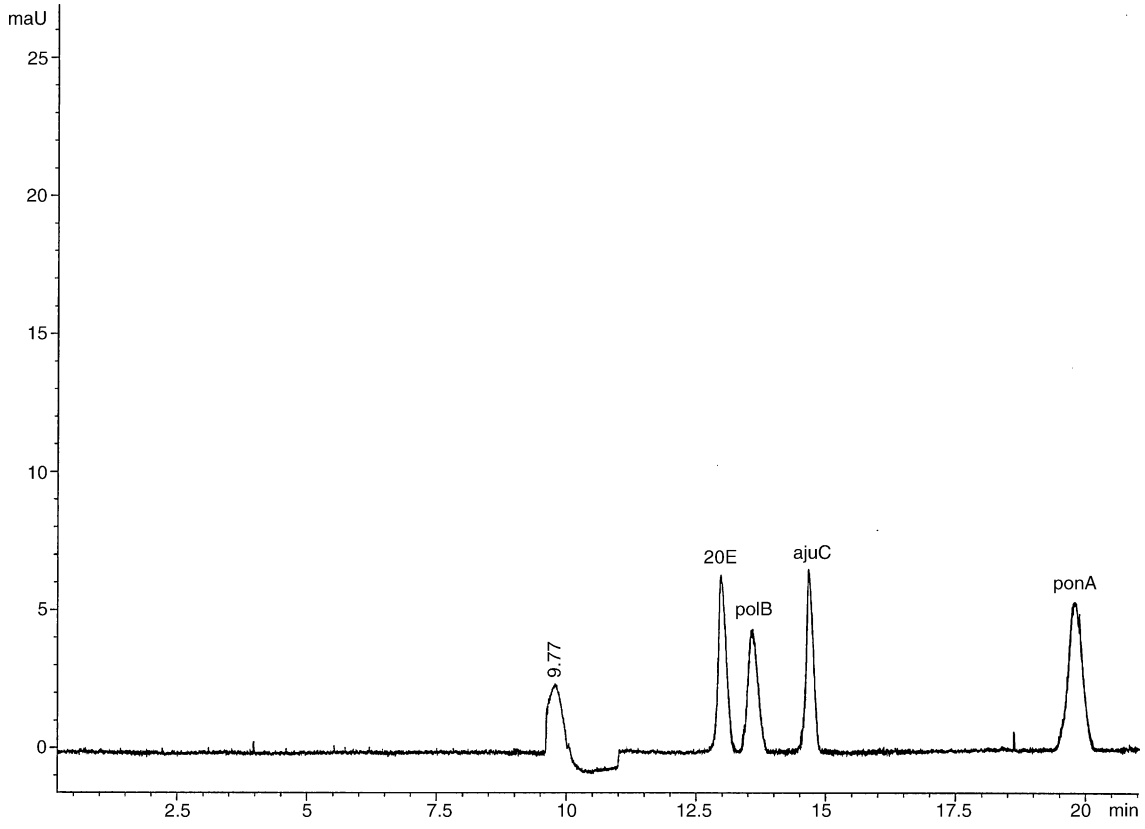

Fig. 2. Optimized electropherogram of selected standard ecdysteroids (70 μg/ml each analyte). Electrophoretic conditions: 50 mM SDS–SC in the ratio of 1:1; 50 mM sodium borate buffer (pH 9.0); 10% (v/v) 2-PrOH. Peak identity and retention time: EOF (9.77 min), 20E (12.98 min), polB (13.55 min), ajuC (14.73 min) and ponA (19.81 min). Other conditions: fused-silica capillary total length, 64.5 cm (effective length, 56 cm); 50 μm I.D.; hydrodynamic injections of 12 s at 50 mbar; temperature, 20 °C; voltage, 20 kV; detection wavelength, 254 nm.

3.2. Method optimisation

The optimisation of the chosen ecdysteroids separation was performed with the aim of develop a general applicability MEKC method. Particular attention was nevertheless focused on the baseline separation of these structurally similar neutral steroids (20E, polB, ajuC and ponA) and on the improvement of detection sensitivity in MEKC by on-line sample concentration technique. In order to develop a method able to meet the previous requirements, the effects of the nature and concentration of different anionic surfactants: SC, STC and SDS (singly or mixed), applied voltage, pH buffer value (range, 8.0–10.0), amount of organic solvent (MeOH, EtOH, BuOH, 2-PrOH and MeCN in the range, 5.0–20.0%, v/v) and injection time on the migration times and stacking efficiency of the studied analytes were evaluated.

3.2.1. Surfactant composition and concentration

The nature and concentration of the surfactant is an important parameter controlling the analysis selectivity. When a surfactant concentration in separation solution is higher than the critical micelle concentration, single surfactant molecules aggregate to form micelles. These have a dynamic structure due to the rapid equilibrium between aggregated and individual forms and it is the different interaction between the micelle and the neutral solute that cause the separation. The anionic surfactant sodium dodecyl sulphate is the most widely used for on-line sample concentration technique. During the optimisation of the method we used different kinds of anionic surfactant as pseudostationary phase: sodium dodecyl sulphate, sodium cholate and sodium taurodeoxy cholate over the 25–100 mM concentration range. In our method, a crucial step was the separation between 20E and polB. It was found the separation selectivity was good with 50 mM concentration of SDS, but the peak of 20E overlapped the peak of polB. Besides, a broad peak and a long retention time for the ponA were observed. The use of STC carried out bad results. By using 50 mM of SC an improvement was observed. In fact, the peaks were almost separated and the compound ponA showed a reduced retention time. Consequently, the effects of the mixture of SDS and SC over the 50–100 mM total concentration range and their different ratios (2:1, 1:1 and 1:2) on the separation was investigated. The optimum conditions for separation consisted of 50 mM concentration of SDS–SC in the ratio of one to one dissolved in solution of 50 mM sodium borate buffer adjusting the pH to 9.0 with NaOH 0.1 M and incorporating 2-PrOH (10%, v/v). An SDS–SC mixture concentration above 50 mM did not improved their resolution or stacking efficiency but only extends the analysis time, up to 40 min for the 100 mM concentration. The effect of different surfactants on the separation are illustrated in Fig. 3.

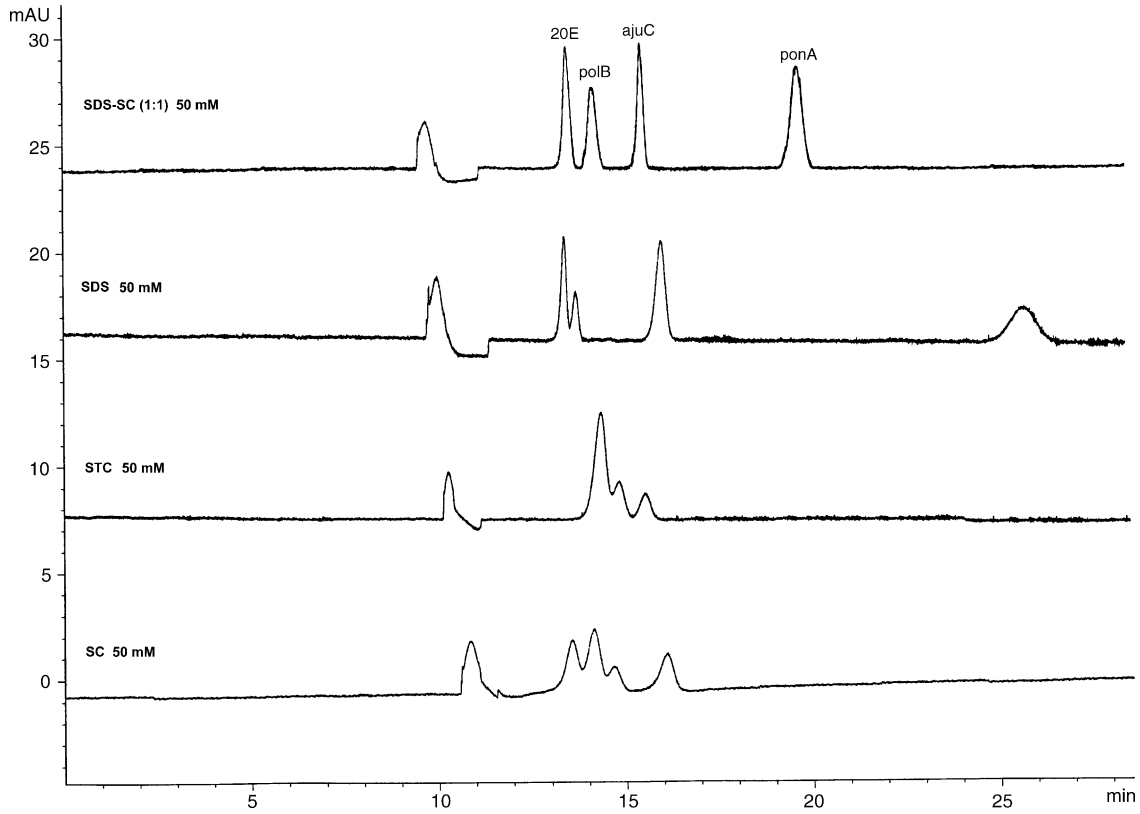

Fig. 3. Effect of surfactant SC, STC, SDS and mixture of SDS and SC (1:1) on the ecdysteroids separation. Electrophoretic conditions: 50 mM concentration of surfactant in 50 mM sodium borate buffer (pH 9.0) and 10% (v/v) 2-PrOH. Others conditions as in Section 2.

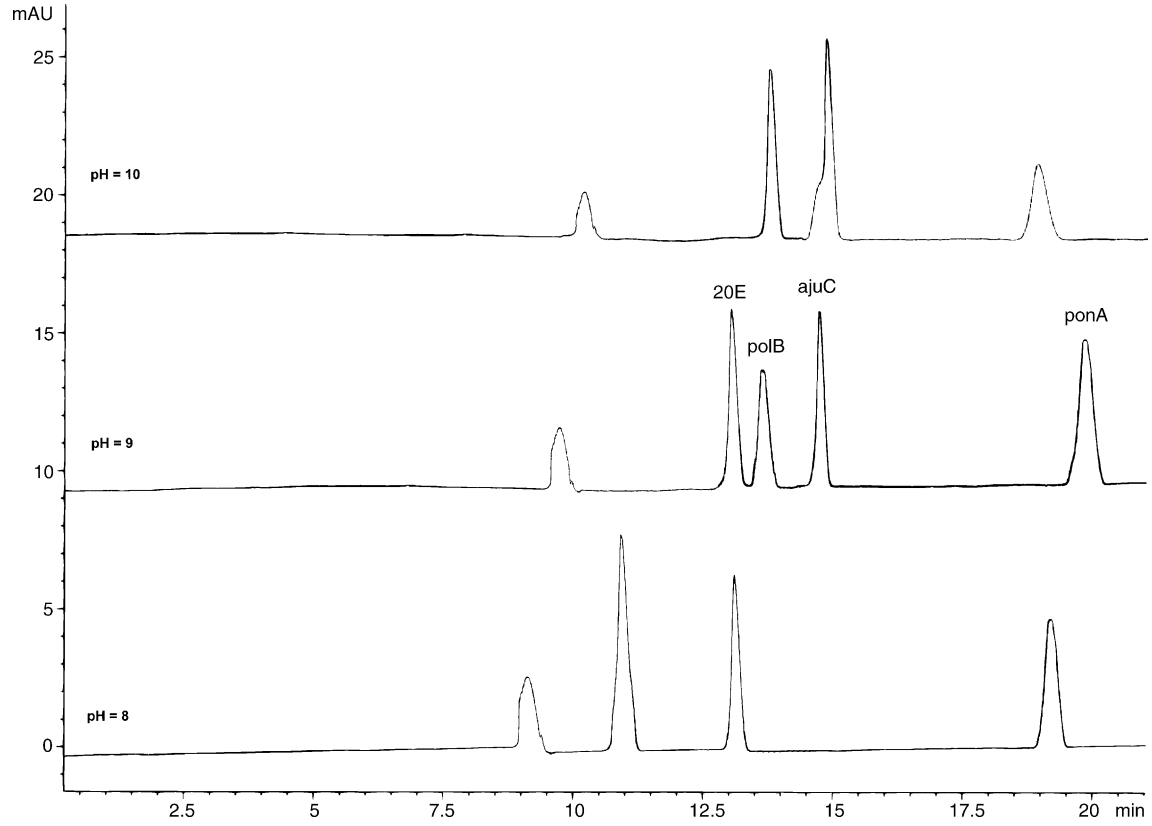

Fig. 4. Effect of pH value on the ecdysteroids separation. Electrophoretic conditions: 50 mM concentration of surfactant SDS–SC (1:1) in 50 mM sodium borate buffer. Others conditions as in Section 2.

3.2.2. Applied voltage

In the electrophoretic capillary methods, high voltage up to 30 kV could be used. Generally, with the increase in voltage the resolution improve. However, very high voltage could cause Joule heating effect if the produced heat would not be effectively dissipated, thence, accumulated heat in capillary disimproved the separation efficiency. Based on experiments, 20 kV was selected as the optimum voltage to accomplish a good compromise between migration times and efficiency of separation. According to the parameters above mentioned, the best resolution was obtained at pH 9.0 with a BGS constituted of 50 mM concentration of SDS–SC in the ratio of 1:1 and 20 kV applied voltage.

3.2.3. Running buffer pH

The buffer pH is a very important parameter controlling the electroosmotic flow (EOF) and the ionisation degree of each analyte. In the usual separation solution in MEKC an high buffer pH such as borate or phosphate is used to generate a substantial EOF. In this methodology, the separation of analytes can be explained by their different interaction with micellar PS phase, their partition on the surrounding aqueous phase and electrokinetic process. In order to improve the separation selectivity and/or the analysis time of studied compounds, the effect of three different pH values (8.0, 9.0, 10.0), employing the same buffer composition (50 mM H_3BO_3–NaOH), were evaluated. For each pH value examined, all neutral steroids showed cathodic migration. The best result was obtained at pH 9.0 because an optimum resolution was achieved. At lower pH (8.0) and higher pH (10.0), no important changes on migration times were observed, but remarkable overimposition between the analytes was showed. These effects are illustrated in Fig. 4.

3.2.4. Organic solvent concentration

The role played by organic solvent is fundamental in CE. Its influence on physico-chemical parameters is decisive for selectivity [34]. Generally, organic solvents are favourably applied to enhance the separation selectivity on the CE analysis: (i) by influencing the mobility of EOF, determined by the zeta potential near the surface on the capillary; (ii) by influencing the viscosity and the dielectric constant of the BGE solution close to the surface; (iii) by changing the effective mobility and the pK_a value of the separated electrolytes; (iv) by increasing the solubility of analytes. During the method optimisation, we evaluated several organic solvents: MeOH, EtOH, BuOH, 2-PrOH and MeCN. The ecdysteroids separation was carried out by trying the aforesaid solvents at different concentrations: 5.0, 10.0, 15.0 and 20.0% (v/v). Initially, using MeOH, EtOH and MeCN large and asymmetric peaks were obtained with high migration time, especially for ponA. An improvement was found by using low concentration of BuOH because an increased resolution between 20E

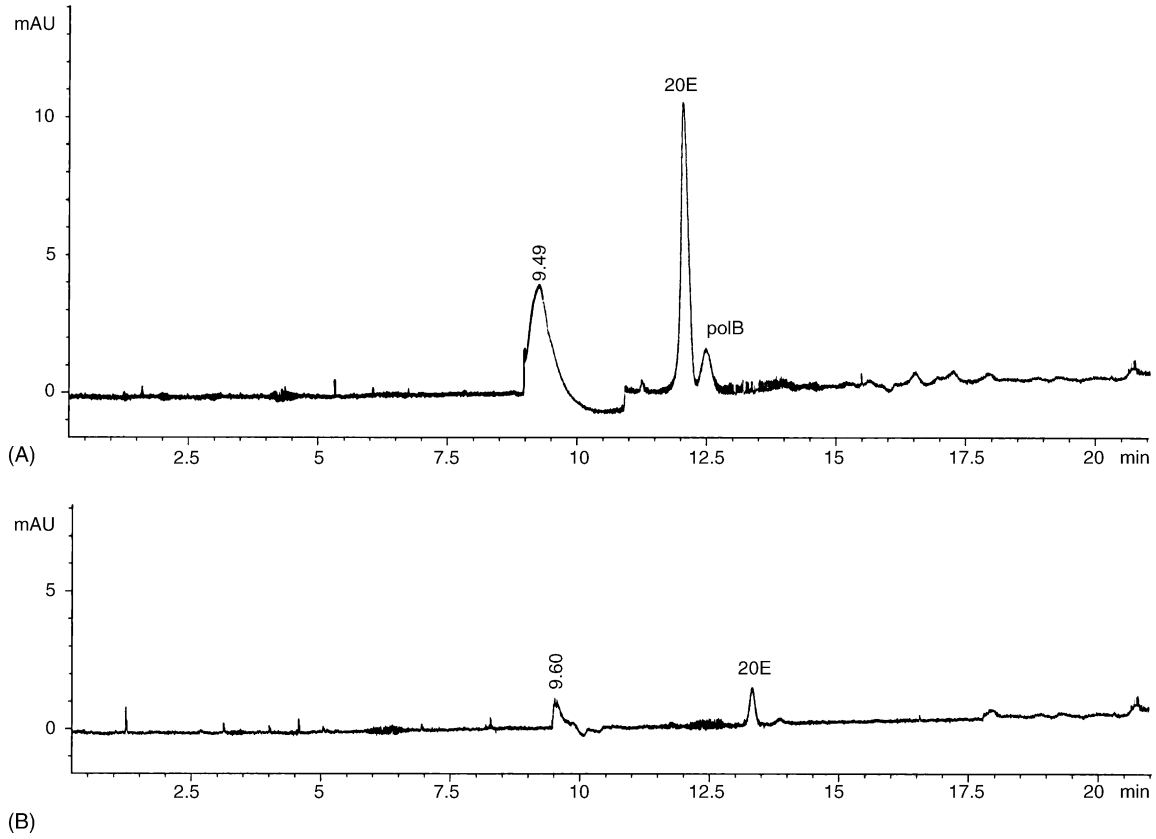

Fig. 5. Comparison of electropherograms showing on-line preconcentration by NSM under optimized condition in a *Pfaffia paniculata* extract. MEKC conditions are the same as in Fig. 2. The representative electropherograms were obtained from: (A) hydroalcoholic extract of plant root of *Pfaffia paniculata* with hydrodynamic injections of 12 s. Peak identity and retention time: EOF (9.49 min), 20E (11.61 min), polB (11.92 min). (B) Hydroalcoholic extract of *Pfaffia paniculata* with hydrodynamic injections of 1.2 s. Peak identity and retention time: EOF (9.60 min), 20E (13.38 min).

and polB with a general reduction of the migration times was obtained. Finally, a concentration of 10% (v/v) of 2-PrOH was chosen as the optimum conditions in terms of resolution, analysis times and peak shape. From these observations, an active role on the separation by organic solvent can be confirmed regarding both the migration times and the peak shape.

3.2.5. Injection time and repeatability

Stacking was performed by injecting sample solutions for a much longer time compared to usual hydrodynamic injection (from 1 to 2 s). The sample solution was hydrodynamically introduced (12 s) on the anodic part of the capillary (50 mbar), then a positive voltage of 20 kV was applied. In our experiments of sample stacking, the injection time was increased from 1.2 s up to 25 s. Both peak height and corrected peak area (peak area divided by the migration time) increased in proportion to the injection time. The 12 s injection was the most suitable in terms of peak shapes. For much longer injection time peaks showed asymmetric shapes. This limitation could be explained by the dispersive effect due to partial mixing on the stacked zones [33]. In order to evaluate quantitatively the sensitivity of enhancement, for each test analyte the degree of stacking was calculated. Quirino and Terabe [29] described the evaluation of stacking efficiency in the on-line concentration technique NSM. In that work, stacking efficiencies in terms of peak height or peak area can be calculated. Also, a stacking efficiency of 10 is comparable to one order of magnitude improvement in concentration detection limit. In our paper, stacking efficiency in terms of peak height (SE_{height}) were computed for each test analyte to evaluate quantitatively the degree of stacking. The found values of SE_{height} of the four ecdysteroids were relatively close and indicate a greater of 10 average of stacking efficiency. All experiments were carried out five times and the calculated average values of SE_{height} are given in Table 1.

Despite the unfavourable effect of mixing due to the difference in local electroosmotic flows between the sample zone and the BGS zone, acceptable repeatability was achieved. The repeatability (RSD) of peak height, corrected peak area and migration time of the studied analytes is reported in Table 1. The RSD ($n = 5$) values obtained for the examined parameters were less than 6% for all test compounds.

3.3. Method validation

The developed MEKC method was validated under the optimised experimental conditions. The selectivity of the method was verified by analysing mixtures of pure and

commercially available standard ecdysteroids. The peak identity for the analyzed samples was confirmed by the migration time values and the on-line recorded UV spectra (DAD). Multiple injections intra-day of a single solution of all ecdysteroids were performed to verify the repeatability of the migration times, peak heights and corrected peak areas (area/migration time). The obtained RSDs, at the level of 70 μg/ml for all the analytes, are summarised in Table 1.

Fig. 2 shows a typical electropherogram of the four standard ecdysteroids mixture separated under the optimum conditions. A baseline separation was obtained for all structurally similar analytes within 21 min. For quantitative applications, the response linearity was verified for all analytes, potential components of plant extracts, measuring the absorbance at 254 nm. The correlation coefficients of the linear regression were greater than 0.99. The values of LOD and LOQ are reported in Table 1.

These data support the suitability of the proposed MEKC method for its application to real samples.

3.4. Applications to a *Pfaffia paniculata* extract

The developed MEKC method was applied to the identification and quantification of ecdysteroids in a sample of *P. paniculata* hydroalcoholic solution. The analysis was performed using the same described conditions for the standard ecdysteroids. Representative electropherogram obtained from the analyzed hydroalcoholic solution using the developed on-line preconcentration method is reported in Fig. 5A. In the analyzed sample, two PEs were found: 20E and polB. The identity of the peaks in the electropherograms was confirmed on the basis of the corresponding UV spectra, which were found to be overimposable to those from standard. Identity of the analytes was also confirmed by spiking experiments. By the quantitative analysis, performed in triplicate, the values of 49.6 (±1.23) μg/ml for 20E and of 10.7 (±1.98) μg/ml for polB, respectively, in the commercial sample have been found.

A usual injection of 1.2 s of the same sample (Fig. 5B) is included for comparison. Without the stacking preconcentration process the detection of the compound 20E is about 10-fold lower compared to the Fig. 5A, while the ecdysteroid polB is no detected. Based on the optimised conditions, the enhancement of sensitivity was demonstrated in a real sample.

4. Conclusion

The reported method is an example of application of on-line sample preconcentration in MEKC on the principle of the NSM, using a mixture of anionic surfactant. An excellent separation can be obtained for structurally similar ecdysteroids compounds such as 20-hydroxyecdysone, polypodine B, ajugasterone C and ponasterone A on a common fused-silica capillary within 21 min. In this study, we demonstrated to obtain about a 10-fold enhancement in sensitivity, relative to a conventional injection, with NSM of neutral analytes. The method was found to be suitable for the determination of specific phytoecdysteroids: 20-hydroxyecdysone and polypodine B in an extract of *P. paniculata*. The proposed methodology, owing to the enhancement in concentration sensitivity and the accuracy and precision demonstrated, can be considered an effective and useful alternative to the conventional MECK or HPLC methods for simultaneous analysis of the described natural compounds.

Acknowledgement

This work was supported by a grant from MIUR, Rome, Italy.

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