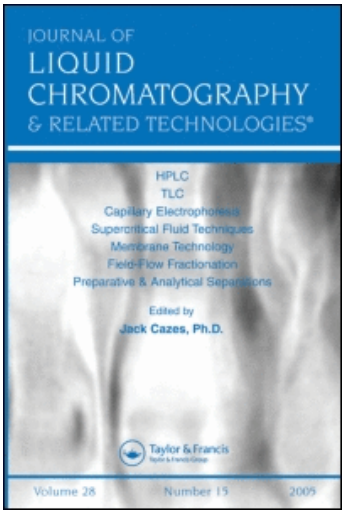

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Optimization of the Separation of Triazines, Metabolites, and Phenylurea Herbicides in Mixture by Reversed Phase Capillary Electrochromatography

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Abstract: The separation of 10 different phenylurea (PHU) and triazine (TRZ) herbicides in mixture was optimized by reversed phase capillary electrochromatography (CEC) by studying the effect of several physico-chemical parameters such as the mobile phase buffer type, pH and concentration, the acetonitrile concentration, and the separation capillary length. Two different buffer systems were investigated, namely ammonium 2-morpholinethanesulfonic acid (MES) and acetate buffers in the pH range 5–7. In MES mobile phases, the triazines herbicides elution order and separation was strongly influenced by the pH. The separation of atrazine and metobromuron compounds was difficult to obtain and was only achieved using a column of 62 cm total length. The separation of all the compounds in mixture was obtained in 5 mM ammonium acetate mobile phase pH 6.0 containing 75% of acetonitrile. Under these conditions, the method was linear in the range 2.5–50.0 μg/mL and exhibited a detection limit of 1.25 μg/mL.

By slightly lowering the mobile phase acetonitrile content, the co-separation of the 10 herbicides with the atrazine *N*-dealkylated metabolites was also successfully achieved.

Keywords: Triazines, metabolites, phenylurea herbicides, capillary electrochromatography

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INTRODUCTION

Modern agriculture is strongly dependent on the use of pesticides to control weeds in crops and to achieve a high yield. Among them, the herbicides are the most important class of agrochemicals, which comprises different groups of both neutral and charged compounds. Due to the increasing application of agrochemicals and due to their toxicity, the assessment of health safety along the entire food chain requires the availability of fast and highly efficient analytical methods for monitoring and controlling their maximum residue limit (MRL) allowed in food matrices regulated by specific national and European legislations.

Due to the use of mixtures of different herbicide compounds in several commercial formulations, the development of analytical methods for their simultaneous separation is of great importance. The recently developed capillary electrophoretic techniques, with their characteristics of high efficiency and resolution power, can be alternative or complementary to the more common liquid chromatography for the analysis of non-polar, polar, and/or thermolabile compounds.

Phenylureas (PHUs) and triazines (TRZs) are important chemicals for the broadleaf and grassy weed control in many agricultural crops, e.g., corn, sugarcane, and sorghum, and in non-agricultural situations. High-efficiency analytical methods are needed for monitoring them in these complex matrices due to their high persistence, together with the corresponding metabolites in the environment and food commodities.

Among the analytical methodologies used for the analysis of agrochemicals, the capillary electrophoretic techniques are increasingly being used in several modes of separation, e.g., MEKC, CE, chiral CZE,[1,2] and capillary electrochromatography (CEC).[2,3]

CEC, a hybrid technique combining both the separation principles of liquid chromatography and capillary electrophoresis, provides unique features in terms of selectivity and separation efficiency and has proven to be very effective in analysing compounds in complex matrices. Furthermore, owing to the presence of a stationary phase in the capillary and the EOF as the driving force, CEC is particularly suitable for separating neutral analytes, as the PHUs and TRZs herbicides, using their different stationary/mobile phase partitioning as separation mechanisms.

As demonstrated in our previous paper,[4] the coupling of the CEC technique with solid phase sample extraction enhances the limit of detection of the method, allowing the determination of the analytes of interest at ppt levels in the environmental matrix. Although some papers report the analysis of PHUs by CEC,[5–7] to our knowledge the separation of TRZs by CEC was still never published.

The aim of this paper was to demonstrate the capability of reversed phase CEC in optimizing the simultaneous separation of four PHUs herbicides,

namely, diuron, isoproturon, linuron, and metobromuron and six TRZs, namely, atrazine, cyanazine, metribuzin, prometryn, simazine, and simetryne, in the presence of triazine *N*-dealkylated metabolites.

The baseline separation of 10 different herbicides in mixture required the careful study of several physico-chemical parameters such as the mobile phase buffer and pH, the mobile phase organic solvent content, and the length of the separation capillary.

EXPERIMENTAL

Instrumentation

Experiments were performed with a Hewlett Packard HP^{3D} Capillary Electrophoresis automated apparatus (Waldbronn, Germany) equipped with a diode array UV detector and external nitrogen pressure (up to 12 bar). The separations were performed on silica fused capillaries, 100 μm I.D., 375 μm O.D. (Composite Metal Services, Hallow, Worcs., UK), fully packed in the laboratory with Lichrospher 100 RP_{18} (5 μm particles) (Merck, Darmstadt, Germany) following the procedure already described.[8] In order to optimize the separation, capillaries of different lengths were prepared and used, namely 31, 37, and 62 cm of total lengths corresponding to effective separation lengths of 23, 29, and 54 cm, respectively. During the run, the capillaries were pressurized at both ends by applying 8 bar from the external pressure and air thermostated at 25°C. The separation was performed in positive polarity mode at 30 kV of applied voltage, using 210 nm as the output UV wavelength. Samples were injected using the high pressure at 12 bar × 3 min followed by mobile phase injection at 12 bar × 0.2 min. Between runs the capillaries were rinsed with the mobile phase at 12 bar × 2 min. To speed up the analysis, the 62 cm capillary was also used in pressure assisted CEC mode by applying, during the run, the high pressure (12 bar) only at the inlet end side of the capillary.

Reagents

Ammonia solution (30%) and glacial acetic acid were purchased from Carlo Erba (Milan, Italy). Acetonitrile and methanol were of HPLC gradient grade from J.T. Baker (Deventer, The Netherlands). 2-Morpholinethanesulfonic acid (MES) monohydrate was from Fluka (Buchs, Switzerland). Double distilled water (Milli-Q, Millipore, Waters Milford, MA, USA) was used for preparation of solutions and for CEC experiments. Atrazine, simazine, cyanazine, metribuzin, prometryn, simetryne, diuron, isoproturon, linuron, and metobromuron herbicides were reference materials for residue analysis purchased from Labor Dr. Ehrenstorfer-Schäfers (Augsburg, Germany).

Atrazin-desethyl-desisopropyl, atrazin-desisopropyl, and atrazin-desethyl were PESTANAL® reference material from Riedel-de Haën (Sigma-Aldrich, Steinheim, Germany). (±)-*trans*-Sobrerol was from Aldrich (Steinheim, Germany). Concentrated analytes solutions (1 mg/mL) were prepared in methanol. Further dilutions were made in water or water/methanol (4 : 1, v/v) solution as subsequently specified.

RESULTS AND DISCUSSION

As a consequence of the successful application of CEC in environmental analysis, this technique was tested for the optimization of the separation of selected PHUs and TRZs herbicides in mixtures in the presence of *N*-dealkylated triazine metabolites for potential multiresidue analysis applications. Based on our previous results,[4] preliminary experiments were performed in ammonium MES or acetate buffer pH 6.0 mobile phases containing acetonitrile in 100 μm I.D. capillary fully packed with ODS 5 μm stationary phase.

On the basis of the physico-chemical properties of the analyzed herbicides (see the chemical structures in Figure 1), the reversed phase CEC seemed to be particularly suitable for their analysis in mixture using the different analytes chromatographic partitioning as the main separation mechanism. With the exception of simetryne and prometryn, which could exhibit a partial positive charge due to to their pKa values,[9] all the analytes were neutral at the working pH range (5.0–7.5) and, therefore, the electrophoretic mobility did not contribute to the separation process.

Figure 2 shows the influence of buffer type in 65% acetonitrile mobile phase separately for PHUs and TRZs herbicide mixture. Under these conditions, the

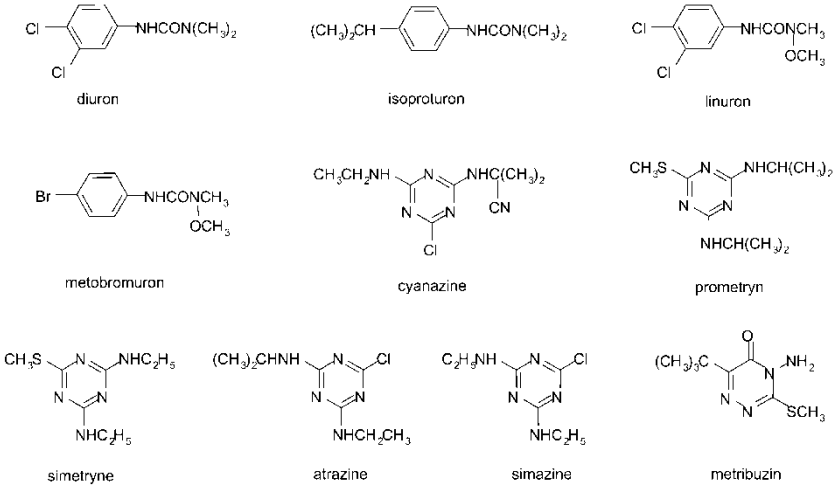

Figure 1. Chemical structures of the analyzed herbicides.

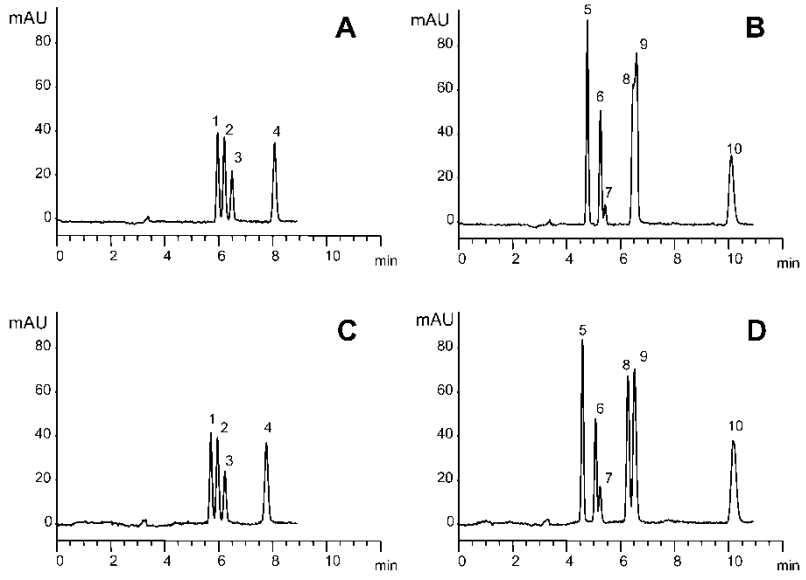

Figure 2. Analysis of separated mixtures of PHUs and TRZs herbicides in ammonium MES (panels A and B) and acetate (panels C and D) mobile phases containing 65% acetonitrile. Capillary: 100 μm I.D., 31 cm total length, 5 μm packed for full length. For other experimental conditions see the experimental section. (1) isoproturon, (2) diuron, (3) metobromuron, (4) linuron, (5) cyanazine, (6) metribuzin, (7) simazine, (8) atrazine, (9) simetryne, (10) prometryn.

PHUs were baseline resolved in both the buffer systems used (Figure 2A and C). The TRZs, instead, showed a strong influence of the buffer type on the separation and particularly the acetate buffer provided better results than the MES (Figure 2D and B, respectively). However, when PHUs and TRZs were analyzed in mixture in the acetate buffer mobile phase (Figure 3), all the analyzed compounds were separated in less than 11 min with the exception of atrazine and metobromuron that were co-eluting under these conditions.

In trying to obtain the complete separation of all the analytes, different physico-chemical parameters were studied as, e.g., the mobile phase buffer pH and concentration, the mobile phase content of organic solvent, the capillary length.

Method Optimization: The Effect of pH

The effect of pH was studied in 65% acetonitrile concentration in the limited range 5–7, according to the buffering capacity range of ammonium MES and acetate buffers and to the need of delivering quite strong electro-osmotic flow.

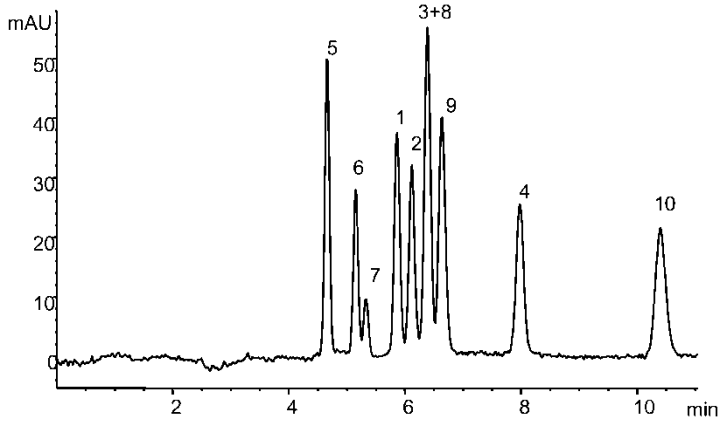

Figure 3. Reversed phase CEC analysis of PHUs and TRZs in mixture. Mobile phase: 5 mM ammonium acetate pH 6.0, 65% acetonitrile. For other experimental conditions see Figure 2.

In ammonium acetate mobile phase, the variation of pH did not exhibit a strong influence on both PHUs and TRZs retention times and separation (Figure 4A and B); however, a slight loss of resolution was observed for TRZs with higher pH.

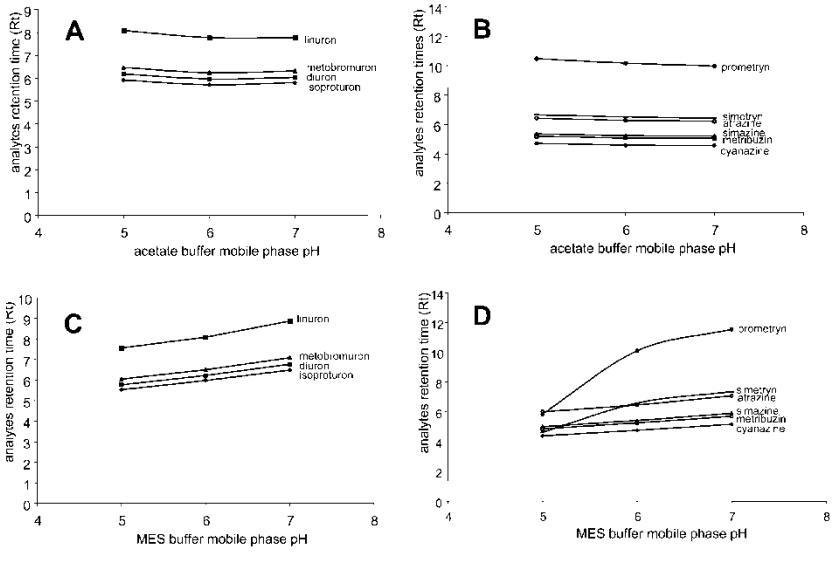

Figure 4. Effect of pH on PHUs (panels A and C) and TRZs (panels B and D) retention time (Rt) in MES and acetate mobile phases containing 65% of acetonitrile.

In MES mobile phase the effect of pH was completely different; particularly for both PHUs and TRZs, the increase of pH from 5 to 7 produced longer retention times (Figure 4C and D), according to previous findings.[8,10] Whereas this effect did not influence the PHUs separation, the TRZs showed an improvement of their resolution at pH 7.0 (Figure 4D).

Considering the final purpose of obtaining good resolution in the shorter analysis time, the best operating conditions for PHUs separation were found in MES at pH 5.0, but comparable separation and analysis time were obtained also in acetate mobile phases at all the studied pHs. The TRZs showed the complete separation in acetate at both the pHs 5.0 and 6.0, but in MES only at pH 7.0.

The different pHs produced the same effect also when the PHUs and TRZs were analyzed in mixture. Comparable results on the separation were obtained in MES at pH 7.0 and in acetate at pH 6.0; however, in acetate mobile phase the analysis time was shorter.

In trying to baseline separate the metobromuron and atrazine compounds still co-eluting under these experimental conditions, the effect of buffer concentration was also studied in MES mobile phase 70% of acetonitrile. The use of MES with respect to the acetate was particularly advantageous for studying the effect of mobile phase buffer concentration due to the low conductivity of the MES buffer.

Method Optimization: Effect of Mobile Phase Buffer Concentration and Organic Solvent

The effect of mobile phase buffer concentration was studied in the range 5–20 mM (final concentration in the mobile phase); more interestingly, under experimental conditions, MES buffer, pH 6.0, 70% acetonitrile where in addition to the co-eluting atrazine and metobromuron compounds, simetryne also showed a partial resolution.

The analytes retention increased with MES concentration (data not shown) and the total analysis time rose from 10 to 15.5 min, according to the lower electro-osmotic flow recognized. In fact, the electro-osmotic flow mobility (μ_{EOF}) decreased from 13.7 to $8.86 \times 10^{-4}\,cm^2/V\,s^{-1}$, from 5 to 20 mM buffer concentration, respectively. This effect positively influenced the separation of the three unresolved compounds improving the resolution of simetryne; however, the atrazine and metobromuron co-eluted also under these conditions. In 10 mM MES, where an acceptable resolution was obtained in reasonable analysis time (less than 13 min), the effect of acetonitrile content was investigated at 60%, 65%, and 70% (v/v).

The effect of the acetonitrile concentration was opposite for PHUs and TRZs herbicides. In fact, PHUs showed an improvement of their separation by lowering the organic solvent content, whereas the TRZs were better

separated with increasing acetonitrile concentrations (data not shown). The optimization of the baseline separation of all the analytes in mixture was, therefore, a difficult task. The acetonitrile content of 70% provided the best results for the analysis of the total mixture. All the analytes, with the exception of atrazine and metobromuron, were separated in less than 10.5 min; however, by comparing the separation obtained in 5 mM acetate pH 6.0, 65% acetonitrile, this mobile phase provided comparable results and was, therefore, selected for further investigation due to its high compatibility with the ESI mass spectrometer. In fact, due to the widely increasing use of liquid chromatography and capillary electrophoresis/CEC in coupling with the high specificity mass spectrometer detectors, the development of analytical methods compatible to this purpose is highly requested.

Further Optimization and Method Linearity

In order to separate the metobromuron from atrazine, the effect of the capillary length was finally studied. Two different capillaries of 100 μm I.D. and 36 and 62 cm of total length were fully packed with the stationary phase and tested for separation. Although the analytes interaction with the stationary phase was stronger in the 36 cm capillary, the separation did not show an improvement under the optimized conditions.

A capillary of 62 cm total length (54 cm effective length) was, therefore, tested for the separation. The choice of this dimension originated from the need to use a capillary length suitable for the future coupling of the optimized CEC method with the mass spectrometer detector. Due to the long capillary length, the acetonitrile content of the ammonium acetate mobile phase at pH 6.0 was increased to 75% to obtain acceptable analysis times. To further speed up the separation, pressure-assisted CEC was also performed under the same experimental conditions.

Figure 5a and b shows the separation of all the analytes in mixture in classical CEC mode (both capillary ends pressurized) and in pressure-assisted CEC (pressure applied at the capillary inlet end only), respectively. In these conditions, the baseline separation of atrazine and metobromuron was finally obtained. The separation of the total mixture was achieved in classical CEC mode in less than 30 min, with peak efficiency values in the range of 83.939–102.080 number of theoretical plates per meter (N/m), with the exception of simetryne and prometryn (peaks 9 and 10, Figure 5), which exhibited lower values, 59.620 and 31.222 N/m, respectively. In pressure-assisted CEC, slightly lower values of peak efficiencies were observed (data not shown).

In order to validate the optimized method, the *trans*-sobrerol pharmaceutical compound was selected as reference compound and the internal standard method was used for quantitation. Under these experimental

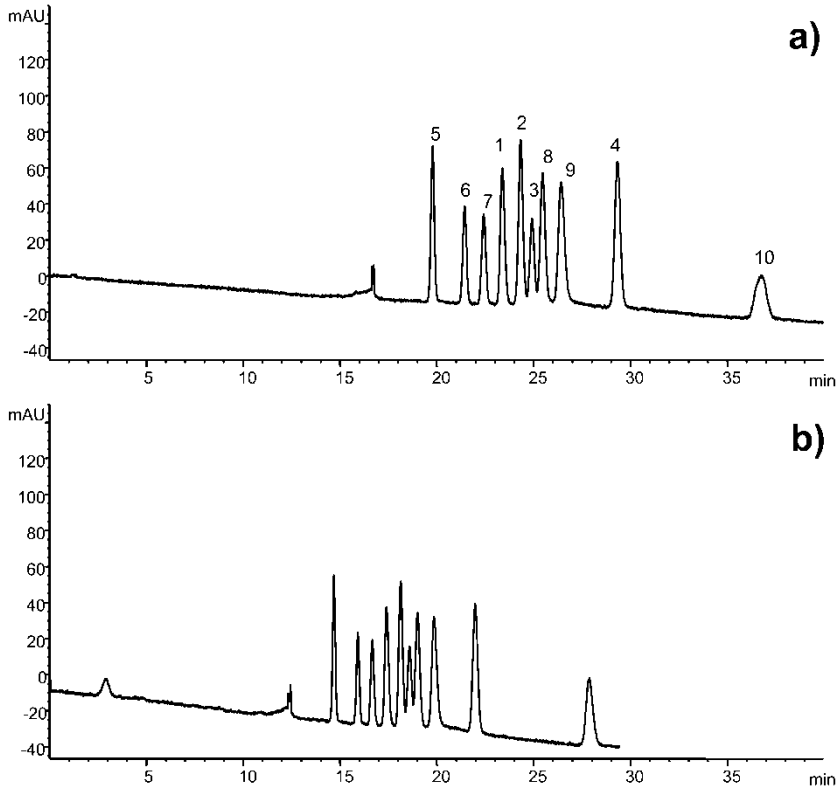

Figure 5. Analysis of PHUs and TRZs herbicides in mixture under the optimum experimental conditions in 62 cm total length packed capillary (effective separation length 54 cm) in (a) CEC and (b) pressure assisted CEC. Mobile phase: 5 mM ammonium acetate pH 6.0 containing 75% acetonitrile. For other experimental conditions see Figure 2 and the experimental section.

conditions, the method provided a limit of detection (LOD) and limit of quantitation (LOQ) of 1.25 and 2.5 μg/mL, respectively, of each analyte standard compound. The method was linear in the range 2.5–50.0 μg/mL (5 calibration levels) giving the relative regression equations and correlation coefficients reported in Table 1.

Co-separation of Herbicides Mixture and Atrazine Metabolites

Under the selected experimental conditions in a 62 cm packed capillary, the mobile phase conditions were suitable for the co-separation of the PHUs

Table 1. Calibration curve data for the studied herbicides

Compounds	Concentration range (μg/mL)	Regression equation	R^2
Cyanazine	2.5–50	$y = 0.0482x - 0.0398$	0.9982
Metribuzin	2.5–50	$y = 0.0266x - 0.0143$	0.9979
Simazine	2.5–50	$y = 0.0261x - 0.0083$	0.9985
Isoproturon	2.5–50	$y = 0.0392x - 0.0133$	0.9984
Diuron	2.5–50	$y = 0.0478x - 0.0319$	0.9982
Metobromuron	2.5–50	$y = 0.0305x - 0.0167$	0.9983
Atrazine	2.5–50	$y = 0.0626x - 0.0294$	0.9985
Simetryne	2.5–50	$y = 0.0695x - 0.0717$	0.9979
Linuron	2.5–50	$y = 0.0537x - 0.0472$	0.9972
Prometryn	2.5–50	$y = 0.0539x - 0.0618$	0.9981

and TRZs herbicides with the *N*-dealkylated TRZs metabolites, namely, the atrazin -desethyl, -desisopropyl, and -desethyldesisopropyl.

The *N*-dealkylation, the primarily metabolic transformation of triazine herbicide in plants, animals, and following microbial and chemical degradation, is not compound-specific since, as with atrazine, other chlorotriazines follow the same biotransformation pathway.[11–13] The availability of analytical methods for the determination of different TRZs in the presence of the relative metabolites is, therefore, important for, e.g., monitoring the environmental pollution and crops contamination and, particularly, for the biological evaluation of human exposure, especially when the simultaneous exposure to different TRZs could have occurred.

Figure 6 shows the simultaneous separation of PHUs, TRZs, and the triazine *N*-dealkylated metabolites in pressure-assisted reversed phase CEC. The metabolites eluted according to their polarity. Due to its highest polarity, the desethyldesisopropyl- metabolite was eluting the most closely to the electro-osmotic flow, demonstrating a light interaction with the stationary phase under the experimental conditions used. In fact, for optimizing their separation, it was necessary to further lower the mobile phase acetonitrile content to 73% (v/v).

CONCLUSIONS

This paper demonstrated the capability of reversed phase CEC in performing the baseline separation of 10 different PHUs and TRZs herbicides in mixtures and in the presence of *N*-dealkylated TRZ metabolites. The high efficiency and resolution capability of CEC permitted obtaining their separation in isocratic mode, avoiding the time consuming gradient elution often necessary in liquid

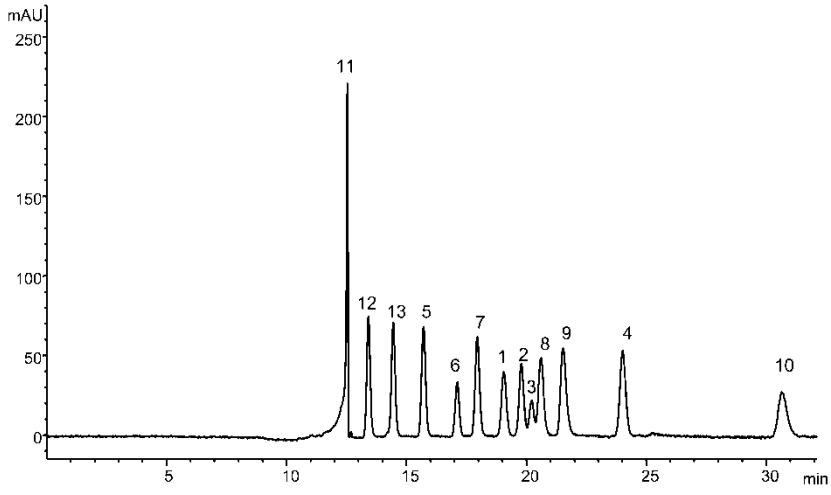

Figure 6. Pressure assisted CEC analysis of PHUs and TRZs in presence of TRZs metabolites. Mobile phase: 5 mM ammonium acetate pH 6.0, 73% acetonitrile. Other experimental conditions as in Figure 5. Peak assignement as in Figure 2. (11) atrazin-desethyl-desisopropyl, (12) atrazin-desisopropyl, (13) atrazin-desethyl.

chromatography. The optimization of the separation, however, showed several difficulties due to the co-elution of some compounds and to the opposite effects observed for the two classes of herbicides of different physico-chemical parameters. Although, two different mobile phase buffers were finally found suitable for their separations, namely ammonium MES and acetate, the latter was selected for its high compatibility with the ESI-MS coupling, and is particularly advantageous for the herbicides monitoring in complex matrices as environment, food, and biological fluids.

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