SIMULTANEOUS SEPARATION OF ERGOT ALKALOIDS BY CAPILLARY ELECTROPHORESIS AFTER CLOUD POINT EXTRACTION FROM CEREAL SAMPLES

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SIMULTANEOUS SEPARATION OF ERGOT ALKALOIDS BY CAPILLARY ELECTROPHORESIS AFTER CLOUD POINT EXTRACTION FROM CEREAL SAMPLES

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Abstract

A new and sensitive analytical methodology for ergot alkaloids determination from cereal samples based on Cloud Point Extraction prior to Capillary Electrophoresis-UV absorbance was developed. The methodology involves extraction under acid conditions and subsequent preconcentration by applying a simple, rapid and environmentally friendly low volume surfactant extraction procedure. After extraction, CE analysis was carried out by performing dilutions on preconcentrated surfactant rich phase, achieving a single peak or simultaneous alkaloids determination. A real preconcentration factor of 22 of total ergot alkaloids was obtained, demonstrating the efficiency of this methodology. The limits of detection were 2.6 and 2.2 μg/kg for ergotamine and ergonovine, respectively. Validation procedure revealed suitable linearity, accuracy and precision. The average extraction and clean-up recoveries were compared with the theoretical values and were better than 92%. This method was successfully applied to the determination of ergot alkaloids in different varieties of commercial flour samples, two grain samples and one of the leading brands cereal based product for infant feeding. The high sensitivity achieved for ergot alkaloids determinations in real samples suggests CPE procedure as an interesting approach to

improve CE-UV visible detection limits. Moreover, the whole process could be considered as a contribution to green chemistry because non-organic solvents were involved, demonstrating its great potential over conventional techniques.

Keywords: Ergot Alkaloids / Ergotamine / Capillary Electrophoresis / Cereals

1. Introduction

Mycotoxins contamination in cereal products has existed for aeons, but it is not until recently that regulatory laws have been developed. Since the discovery of the aflatoxins in the 1960s, regulations have been established in many countries to protect consumers from the harmful effects of mycotoxins that may contaminate foodstuffs. Various factors are important in setting limits for mycotoxins. These include scientific information to assess risk, food consumption data, the level and distribution of mycotoxins in commodities, and certainly, the availability of analytical methodology [1].

Ergot Alkaloids (EAs) are mycotoxins produced by the sclerotium (or ergot) of Claviceps ssp. Chemically, EAs are derived from the ergoline which is a common skeleton of alkaloids, including lysergic acid. More than 100 EAs are known [2] and they are structurally different only in substituents on C8. Among the natural EAs, only ergotamine and ergonovine have important therapeutic applications, employed for migraine attacks and treatment of post-partum bleeding, respectively [3,4].

Claviceps purpurea attacks more than 600 species belonging to the monocotyledons, being Secale cereale (known as rye) one of the most susceptible species [5]. The infected plants develop sclerotia and when these are harvested with grain, seed or grass may result in EAs contamination of cereal products and foodstuffs causing ergot poisoning (known as

ergotism) in human and animals. Ergot cannot be controlled with fungicides [6] therefore cereals require strict clean-up procedure before its consumption. The symptoms of this disease are a feeling of itchy and burning skin, hallucinations, suppression of lactation, hypersensitivity; causing convulsions, ataxia, gangrene, abortion and even death.

The major Latin American agricultural crops are highly susceptible to fungal contamination and therefore, to mycotoxin pollution. The local production of grains must satisfy the requirements of MERCOSUR normative, which is the most applicable for Latin America countries. However, limits of EAs in cereals are not well established yet in this normative [7]. Considering the structurally similarity of EAs and their frequent low levels content in the samples studied, more precise, selective and sensitive analytical methods are required.

Several determination methodologies have been developed for detecting and quantifying EAs in cereal products [3,8,9]. Analysis for EAs is most commonly conducted by HPLC combined with UV or fluorescence detection [2,10]. However, an exhaustive clean-up and extraction/preconcentration procedure is required before analysis, which commonly involves the use of undesirable toxic solvents.

Capillary Electrophoresis (CE) analysis offers some advantages over chromatography, such as low sample and reagents consumption, reduced analysis time, demonstrating its great potential for a wide range of molecules. However, depending on the concentration of the analytes in the studied samples, sensitivity in CE occasionally needs to be improved [11-21]. Frach and Blaschke [22] have demonstrated the efficiency of CE to separate EAs present in sclerotia but the limitation of the method´s sensitivity was not enough for direct cereal analysis.

Cloud Point Extraction (CPE) is one of the non-polluting phase separation techniques using surfactant at concentrations higher than its CMC (critical micelar concentration). The success of organized media in separation and preconcentration science is based on their selective solubilization of analytes. Typically, analytes are extracted from aqueous solutions into micelles. Afterward, the change on the experimental conditions that promotes the phase separation leads to a surfactant rich phase with concentrated analytes on the one hand, and aqueous solution saturated with surfactant monomers on the other hand.

Aqueous/surfactant rich phase volume ratio needed is typically large, indicating the high efficiency of this technique. In our previous works, CPE has proved to be efficient for monitoring drugs level in biological samples. The most important advantage of CPE is that only a small amount of surfactant is required and consequently the procedure is less expensive and more environmentally friendly than other conventional extraction techniques such as liquid extraction and solid-liquid extraction[19,20,23-25].Moreover, CPE offers the possibility of combiningextraction and preconcentration in one step.

In this paper, EAs contained in cereal samples have been preconcentrated by CPE procedure into the surfactant rich phase and have been separated using CE. The non-ionic surfactant employed in the preconcentration step contributed then to the separation of EAs in CE. Furthermore, depending on the dilution of surfactant rich phase and the presence of additives, EAs could be determined either as total content (expressed as ergotamine) or as individual alkaloid. Non-polluting and non-toxic reagents were involved in the whole analysis, including sample pre-treatment, preconcentration procedure and finally in separation step. Also, the combination of low reagents consumption techniques CPE-CE, make this methodology, agreen chemistry alternative to the conventional analysis.

2. Material and methods

2.1. Instrumentals

The CE system consisted of a Beckman Coulter P/ACE MDQ instrument (Beckman Instruments, Inc. Fullerton, CA) equipped with a diode array detector and a data handling system comprising an IBM personal computer and P/ACE System MDQ Software. A pH meter (Orion Expandable Ion Analyzer, Orion Research, Cambridge, MA, USA) Model EA 940 with combined glass electrode was used for monitoring pH adjustment. A centrifuge ROLCO (Buenos Aires, Argentina) was used for accelerate phase separation in CPE.

2.2. Reagents

All chemicals used throughout the experiment were of analytical reagent grade. Solvents employed for CE were of HPLC grade. Sodium dihydrogenphosphate (Merk Darmstadt, Germany) and phosphoric acid (Merk Darmstadt, Germany) were used for background electrolyte (BGE) preparation. β-cyclodextrin (β-CD, Sigma-Aldrich, Burghausen-Germany) was used as a BGE additive.

Powder of standard ergotamine tartrate was kindly provided by lab. Andrómaco S.A. (Bs. As., Argentina) and ergonovine was acquired from lab. Biol (Bs. As., Argentina) in ampoules labeled as containing 2 mg/mL.

PONPE 7.5 (polyoxyethylene(7.5)nonylphenylether, was acquired from Tokyo Kasei Industries (Chuo-Ku, Tokyo, Japan). Sodium tetraborate was acquired from Mallinckrodt (Chemical Works, New York, USA).

The buffers pHs were adjusted using HCl (Merk Darmstadt, Germany) and NaOH (Merk Darmstadt, Germany).

2.3. Standards and assays solutions

The phosphate buffer stock solution was prepared by weighing 0.24 gsodium dihydrogenphosphate and made up to 50 mL. The BGEs used were prepared daily by appropriately diluting the stock solution. The BGE containing β-CD was prepared by dissolving 0.28 g of β-CD with the corresponding BGE and made up to 25 mL.

The standard solution of ergotamine was prepared by dissolving the drug powder in ultra-pure water up to a concentration of 100 μg/mL and stored at 5ºC in an amber flask. The standard solution of ergonovine was prepared by dilution of the ampoule content. The assay solutions were obtained by appropriate dilution from stored standards before analysis.

The extracting solution was prepared by mixing 10.0 g PONPE 7.5 with 40 mL ethanol and made up to 100 mL with ultra pure water.

2.4. Sample preparation and CPE procedure

Several commercial cereal samples were acquired from Argentine local stores. These include six varieties of flour samples (rye, wheat, oat, rice, soybean and corn), two grain samples (wheat and oat), and one of the leading brandsof cereal based products for infant feeding (claimed composition: rice, corn, wheat, oat, barley, sugar and additives).

In the case of flour varieties, a statistical sampling procedure was applied to the bulk and packed commercial products to obtain reduced and representative samples for the analysis.The same procedure was applied to the grains and crushed grains studied. Respect to the cereal based product for infant feeding, the chosen brand is commercialized as 350 g of net weight. Therefore, as with other packed products, the sampling was performed by only mixing and reducing its content for assay.

For sample clean-up procedure, 5.0 g of dry samples were weighted and transferred to 50 mL centrifuge tubes. The tubes were then made up with diluted HCl ($1x10^{-4}$M) and shaken for 15 min. After centrifugation for 15 min (1500 rpm), the supernatants were collected and filtrated through a filter paper into flasks and then re-filtrated through a 26 mm syringe filter (Microclar^{TM}, 0.45 µm porosity). The final filtrates were collected for assays.

For CPE procedure, in a set of 10.0 mL centrifuge tubes, 8.0 mL of aqueous extracts were added in each tube with 1.0 mL of buffer tetraborate ($1x10^{-2}$M, pH 8.5) and 0.3 mL of extracting solution and made up to 10.0 mL with ultra-pure water. The cloudy phenomenon occurs instantaneously at room temperature after mixing. In order to accelerate the phase separation, the tubes were centrifuged for 15 min, leading to a small volume of surfactant-rich phase at the bottom of the tube. The aqueous supernatant was removed by a micropipette and the surfactant-rich phase was diluted with methanol (2:1 and 1:1 surfactant rich phase/methanol) for total EAs or single EAs CE analysis.

2.5. Electrophoretic conditions for the assay

The fused-silica capillaries were obtained from MicroSolv Technology Corporation with the following parameters: 60 cm total length, 50 cm effective length, 75 µm ID, 375 µm OD. The temperature of the capillary and samples was kept at 35ºC. Samples were pressure-injected at the anodic side at 0.5 Psi for 5 seconds. A constant voltage (25 kV) was used for all experiments. UV absorbance at capillary outlet was employed as detection system, at wavelength of 200 nm.

For determination of total EAs, the BGE was an aqueous solution of sodium dihydrogenphosphate ($4x10^{-2}$M), adjusted to pH 4.0 with phosphoric acid ($5x10^{-2}$M).

For EAs separation, the run buffer was an aqueous solution of β-CD ($1x10^{-2}$M) with sodium dihydrogenphosphate ($4x10^{-2}$M), adjusted to pH 4.0 with phosphoric acid ($5x10^{-2}$M).

3. Results and discussion

3.1. EAs extraction parameters

One important characteristic of EAs is the alkalinity of N6 with pKa values of the protonised EAs ranging from 5-7.4. Thus EAs are positively charged in acidic solutions and neutral at higher pH values. Most of the clean-up processes are carried out by liquid/liquid partitioning at alternating acidic and alkaline conditions [4-8], consuming high quantities of organic solvents.

To achieve EAs extraction from cereal samples, a phosphate/phosphoric acid solution (pH 3.5) was employed. After removal of solid remains, the pH of the solution was changed to alkaline condition, thus, the uncharged form of EAs prevails (reaching the maximum at pH 8.5) facilitating the partitioning process into the hydrophobic micellar core in CPE procedure.

Non-ionic surfactant PONPE 7.5 (Fig. 1) was selected for CPE because of some experimental advantage such as low critical cloud point and low UV-Visible signal background [23,24]. In order to study the influence of experimental parameterson CPE, a systematic study was applied for each parameter. This consisted of varying one parameter every time keeping the rest constant. The results were presented in Table 1. The small and compact surfactant rich phase resulted from phase separation, plus high affinity of this surfactant to organic molecules, led to a high recovery factor and therefore great extraction efficacy.

After phase separation, the surfactant rich phase is adequately fluidified in order to facilitate its transference to a vial and the injection into the capillary.

3.2. EAs separation by CE

For all samples, before CE analysis, the obtained gel-state surfactant rich phase was fluidified with methanol. Depending on dilution proportions, different peak resolution of EAs was observed using sodium dihydrogenphosphate (4x10-2 M, pH 4.0) as BGE (Fig. 2).

In the first instance, dilution of 2:1 (surfactant-rich phase/methanol) led to a large single peak of EAs without separation between alkaloids (Fig. 2a). Presence of EAs was observed for rye and oat flour, as well as in the case of the product for infant feeding while the level of EAS in the rest of studied samples was undetectable (Table 2).

In this condition, an effective Preconcentration Factor (Pf) of 22 was obtained comparing the corrected area of the single peak in electropherograms with and without CPE treatment (Fig. 3). This result was in accordance with the theoretical approach, using equation of Pf = Vi/Vs.Df = 24 (Where Vi= initial sample volume; Vs= surfactant rich phase volume; Df= Dilution factor). By comparison between effective Pf and theoretical Pf, we can calculate a recovery factor of 92%.

An interesting separation phenomenon was observed when the concentrated surfactant rich phase was injected at different dilutions with methanol. When the surfactant rich phase was gradually diluted in order to permit the injection into the capillary, EAs begun to separate without any additives in the run buffer, reaching the maximum separation at a dilution factor of 1:1 (Fig 2b). Surprisingly, the EAs partial separation obtained was only due to the dilution of the surfactant rich phase demonstrating the PONPE contribution to this process.

Moreover, the effect of PONPE 7.5 on EAs separation by CE was evidenced when the total separation of EAs was achieved adding β-CD (1x10-2 M) to BGE (Fig. 2c). In the presence of PONPE 7.5, the β-CD concentration needed to achieve the total EAs separation was relatively lower in comparison to research conducted by Frach and Blaschke [22], in which the total 9 EAs contained in the fungus sclerotia sample were separated using a mixture of β-CD (2x10-2 M), γ-CD (8x10-3 M), urea (2 M) and 0.3% poly(vinyl alcohol) in phosphate buffer.

The electrophoretical separation of EAs could be attributed to an association formed between monomers of PONPE 7.5-EAs that cause changes in the electrophoretic mobility of the analytes along the capillary. When β-CD was added to BGE itcould have enhanced the CE separation efficiency by differential affinity between EAs with PONPE 7.5 and β-CD. Therefore, the competition to form PONPE 7.5-EAs and β-CD-EAs complexes along the capillary,can conduct the CE separation of compounds with slight structural differencesuch as EAs.

The effect of additives to the separation process has been widely described in several researches [26], such as the effect of ion-pair formation and Micellar Electrokinetic Chromatography (MECK). Though, in this method, the surfactant is present in the capillary only in the sample plug zone, ina concentration level lower than its Critical Micellar Concentration (CMC). Therefore, further studies are required to confirm the exact mechanism of this phenomenon.

In this work, the simultaneous EAs separation from rye flour samples was achieved with a surfactant rich phase dilution of 1:1 and a BGE composed by β-CD (1x10-2 M) and phosphate buffer (4x10-2 M, pH 4.0) (Fig. 4). The use of single β-CD at working concentration avoids the problem of reagents solubility and solution stability presented

when the mixture of β-CD and γ-CD was employed at high concentrations. In Fig. 4, the presence of ergonovine and ergotamine was confirmed by standard addition. Furthermore, the total CE analysis time was achieved within 9 min, considerably faster than the mentioned CE methodology or any other chromatographic analysis.

3.3. Validation of CE methodology

The precision expressed by relative standard deviations (RSDs) of the migration times and corrected peak areas, was studied with consecutive repeated injections on the same day (repeatability or intra-day precision) and on three different days (intermediate precision or inter-day). The repeatability of the analytical procedure was evaluated by triplicate injections of samples of rye flour spiked with ergotamine and ergonovine. The precision for total EAs determination (expressed as ergotamine) was less than 0.7% for the migration time and 1.1% for the corrected peak area. In the case of individual EAs determination, the precision for the corrected peak areas was less than 1.5% and 2.0% for intra and inter-day, respectively.

The accuracy, in terms of recovery, was verified by applying the proposed method to the rye flour samples and the average concentrations determined for ergotamine were taken as base value. Each dilution was injected in triplicate. These dilutions were spiked with known quantities of ergotamine maleate. The recovery was calculated as: 100 [(found value - base value) /added value]. The results showed good recovery (Table 3), under the established experimental conditions, the results ranged from 102.5% to 104.6% recovery.

Specificity of the method was investigated by both peak purity and spiking experiments with pure standard compounds. Peak purity was evaluated by means of the

P/ACE System MDQ Software. There appeared to be no interference from sample constituents; also, this is in agreement with the recovery test results.

Linearity of the method was evaluated preparing a mixed stock solution containing ergotamine and ergonovine. Sequential dilutions were performed and obtained solutions were added to the rye flour sample after CPE procedure; then they were injected (n=6) in triplicate and the corrected peak areas used to plot calibration curves. The calibration equations were obtained by the least-squares linear regression method and used for unknown concentrations calculation. The analytical values obtained are shown in Table 4.

The amount of standard, which could be detected with a signal-to-noise ratio ≥3 was considered to be the LOD. The LOQ was calculated as the analyte concentration that can be accurately and reliably determined with a signal-to-noise ratio ≥10.

LODs and LOQs were evaluated based on the signal background obtained with the analysis of a diluted mixed standard solution (n =6) (Table 4).

Moreover, robustness of this methodology was checked by varying slightly different CE experimental conditions, such as T°, pH, buffer concentration, surfactant-dilution and injection time. Apart from pH and buffer concentration which affected slightly separation profile varying their values within the studied range, the rest parameters affected significantly to peak resolution (peak overlapping).

3.4. Applications

The developed method has been applied to determine the total EAs content in cereal samples (expressed as ergotamine) and to quantify ergotamine and ergonovine individually, both with clinical and toxicological properties. The results are summarized in Table 2. In Fig. 4 the preconcentration efficiency and recovery of this methodology by standard

addition of ergotamine to rye flour samples was demonstrated. This methodology has been validated and the results were also compared with an official method (UV-Visible) (Table 3).

4. Conclusion

The proposed method provides agreenand sensitive CPE-CE methodology for EAs determination in cereal samples. The simple and effective sample clean up, combined to the high extraction efficiency of CPE using PONPE 7.5 has the advantage ofbeing an environmentally friendly methodology with respect to the traditional EAs analysis. In this work, we attempted to contribute to green analytical chemistry development, avoiding the use of high pollutant solvents. The potentiality of this method resides in obtained results, a real preconcentration factor of 22 was obtained for CPE procedure, indicating the efficiency of this extraction methodology. Moreover, depending on the dilution factor of the surfactant-rich phase and presence of additives in BGE, EAs in cereal samples can be quantified as total EAs (expressed as ergotamine) or as individual alkaloids. For assayed samples, only rye flour contains high EAs concentration level, comparable to those reported from European countries (231 μg/Kg of total EAs).

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Figures Captions

Figure 1. Molecular structure of PONPE 7.5.

a) Micelle of PONPE 7.5; b) Monomer of PONPE 7.5.

Figure 2. Electropherogram of rye flour sample after CPE procedure at different CE experimental conditions.

a) Dilution of surfactant rich phase with methanol (2:1); b) Dilution of surfactant rich phase with methanol (1:1) and c) Dilution of surfactant rich phase with methanol (1:1), addition of β-CD ($1x10^{-2}$ mol/L) in BGE, where peak 1=ergonovine and peak 2=ergotamine. Conditions: BGE, phosphate buffer ($4x10^{-2}$ mol/L, pH 4.0), 25 kV applied voltage, 35ºC capillary temperature, 35ºC sample temperature; hydrodynamic mode sample injection, 0.5 psi during 5 s; detection by DAD at 200 nm.

Figure 3. Single peak of total EAs before (a) and after (b) CPE procedure performed on rye flour sample.

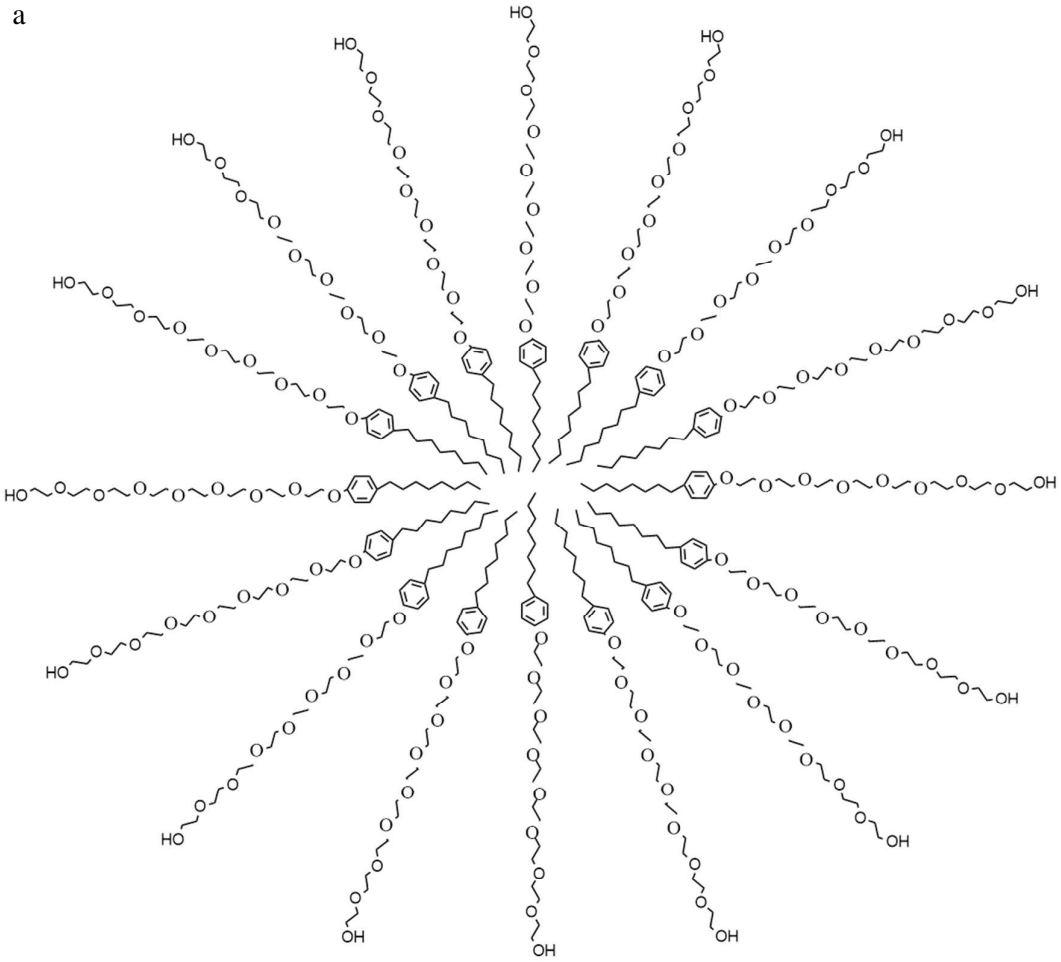
a) Sample: water extract of rye flour (5.0 g); b) Sample: surfactant rich phase diluted of (a) diluted (1:1) with methanol. Conditions: idem Fig 2.

Figure 4. Total separation of EAs in standard addition method performed on rye flour sample.

a) Electropherogram without addition of standard; b) with addition of standard (ergotamine maleate, 2.5 μg). where peak 1 and 2= ergotamine. Conditions: BGE, phosphate buffer ($4x10^{-2}$ mol/L, pH 4.0), β-CD ($1x10^{-2}$ mol/L), 25 kV applied voltage, 35ºC capillary temperature, 35ºC sample temperature; hydrodynamic mode sample injection, 0.5 psi during 5 s; detection by DAD at 200 nm.

Fig 1.

a



b

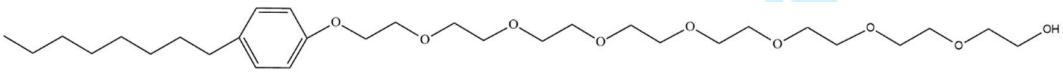


Fig 2.

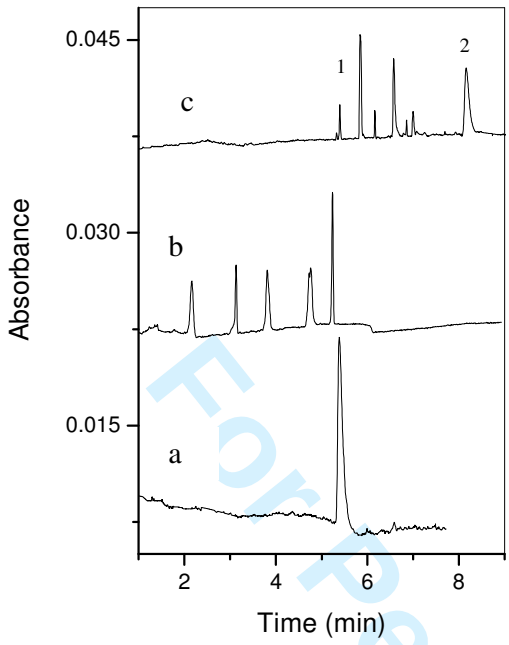


Fig 3.

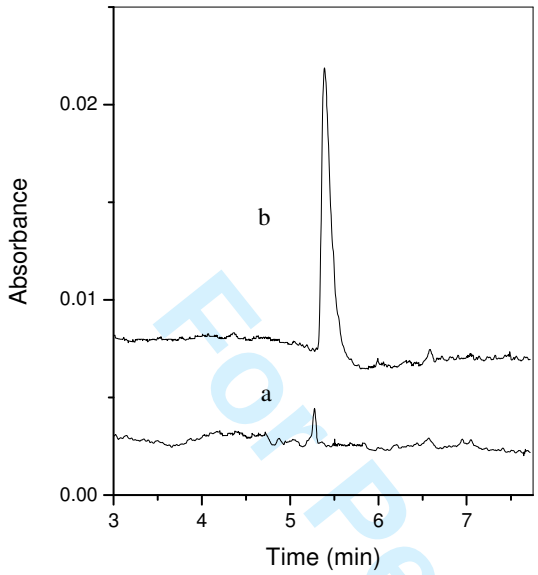


Fig 4.

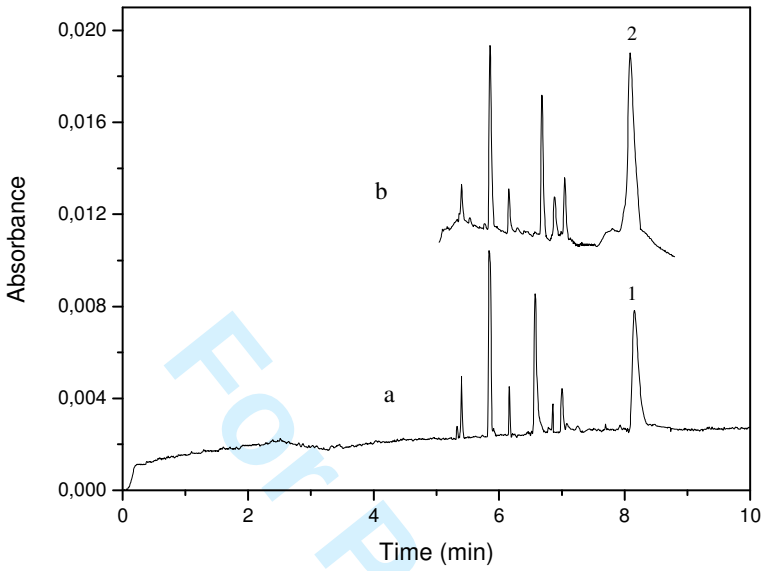


Table 1: Experimental conditions of CPE.

Parameter	Studied range	Optimal condition
$C_{\text{PONPE 7.5}}$ (w/v)	0.05-0.50%	0.20%
pH	3.5-13.0	8.5
C_{buffer} (mol/L)	$5\text{x}10^{-4} - 5\text{x}10^{-3}$	$2\text{x}10^{-3}$
Centrifugation time (min)	5-20	15

Table 2. Comparison of results obtained from the present methodology to an official method, applied to spiked rye flour.

Conc. Added (µg/mL)	[a]Capillary electrophoresis (µg/mL)	Recovery ± RSD (%)	[b]UV-vis spectrophotometry (µg/mL)	Recovery ± RSD (%)
--	230±1.5	--	225±3.0	--
200	431±2.0	100.5±1.0	434±2.5	104.5±1.3
300	529±1.0	99.6±0.4	533±3.0	102.6±1.0

[a]This methodology; [b]Official methodology

. Table 3. Analytical figures of CPE of EAs prior CE analysis

Analytical parameters	Total EAs[a]	Ergotamine	Ergonovine
LOQ (µg/Kg)	19.43	8.25	7.11
LOD (µg/Kg)	5.83	2.57	2.20
Linearity (µg/Kg)	20-400	8-200	7-200
Linear equation[b]	$P=4.3x10^{-5}C+5x10^{-3}$	$P= 5x10^{-5}C+1x10^{-1}$	$P= 7x10^{-5}C+1x10^{-1}$
Correlation coefficient	0.998	0.997	0.996

[a] Expressed as ergotamine tartrate.
[b] Where P is the peak area ratio, C is the concentration of the drug in and r is the correlation coefficient

Table 4. CE analysis of real cereals samples

Cereal samples	Ergotamine (μg/Kg)	Recovery (%)	Ergonovine (μg/Kg)	Recovery (%)	Total EAs[*1] (μg/Kg)	Recovery (%)
[*2]*Flours*						
Rye	77	98	12	96	230	96
Oat	52	89	-	94	143	92
Wheat, corn rice, soy	-		-		undetectable	
[*3]*Grains*						
Oat, wheat	-		-		undetectable	
Cereal product for infant feeding	-	88	-		63	91
[*4]Other varieties of cereals	-		-		undetectable	

*1 Expressed as ergotamine.
*2 *3 different trademark from each varieties.
*4 Barley, sorghun, lentil, etc.