JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of Phytohormones of Environmental Impact by Capillary Zone Electrophoresis

A. SEGURA CARRETERO,* C. CRUCES-BLANCO, M. SORIANO PEÑA, S. CORTACERO RAMÍREZ, AND A. FERNÁNDEZ GUTIÉRREZ

Department of Analytical Chemistry, Faculty of Science, University of Granada, Granada, Spain

A test mixture of five phytohormones [naphthaleneacetic acid (NAA), naphthoxyacetic acid (NOA), indoleacetic acid (IAA), indolebutyric acid (IBA), and indolepropionic acid (IPA)] was investigated. These compounds were cleanly separated with good resolution by capillary zone electrophoresis with a UV diode array detector using 20 mM sodium phosphate buffer (pH 7.25). The lowest detection limit was obtained for IPA (0.45 mg L^{-1} or 0.005 mg kg^{-1}) and the highest for NAA (1.04 mg L^{-1} or 0.014 mg kg^{-1}). The method has been applied for tomato samples fortified with the five phytohormones using a liquid−liquid extraction procedure, obtaining recovery percentages ranging from 91 to 109.0%.

KEYWORDS: Capillary zone electrophoresis; phytohormones; tomatoes

INTRODUCTION

Liquid chromatography (LC) and gas chromatography (GC) are the main workhorses in agricultural analytical laboratories, but in recent years, capillary electrophoresis (CE) has become a versatile and powerful tool for numerous types of analysis (*1-4*), especially for compounds unstable at higher temperatures.

CE is a powerful microanalytical technique based on the electrophoretic separation in narrow capillaries. The fast speed of analysis, high resolution, and sensitivity make CE an attractive method to separate a wide range of compounds. Initially, only charged analytes were studied by the capillary zone electrophoresis methodology (CZE) (*5*), but the high separation efficiency of CE became accessible also for the separation of neutral compounds with the introduction of another mode of operation being called micellar electrokinetic capillary chromatography (MEKC) (*6*). The high-resolution separation power of both CZE and MEKC has permitted their application to many charged and uncharged analytes, including food products (*7*, *8*), industrial and drug analysis (*9*, *10*), or substances of agricultural interest, such as pesticides (*11*, *12*).

Application of CE for the analysis of agrochemicals is gaining in popularity. A number of reports have been published in past years describing the use of CZE for the separation and detection of several pesticides including herbicides (*13*−*17*) and insecticides (*18*−*21*) and demonstrating the potential application of CE techniques to environmental issues.

Phytohormones are substances that, at low concentration, influence plant growth and differentiation. Formerly referred to as plant hormones or phytohormones, these terms are now suspect because some aspects of the hormone concept, notably action at a distance from the site of synthesis, do not necessarily apply in plants (*22*, *23*). Hormonal involvement in physiological processes can appear through several distinct manifestations (as environmental sensors, homeostatic regulators, biotime adjusters, etc.) (*24*). In addition, it is known that phytohormones play a role in sex expression in plants (*25*).

In agriculture phytohormones are used to influence the growth of cultivated plants and especially to prevent fruit fall. Their toxicity decreases 3−4 weeks after application, so fruit treatment and picking are especially regulated in all countries.

Phytohormones are recommended for the alteration of the hormonal system in women and have a regulator effect, which increases the activity of the estrogens when the body needs them. However, if these cells are present in an excess, phytohormones reduce this amount. Phytohormones are used in treatment against cancer (*26*−*28*).

The problems associated with analyses for these compounds are always related to the problem that, in most instances, the history of the product is unknown, so analytical methods that permit the identification and determination of all of the residues present are required.

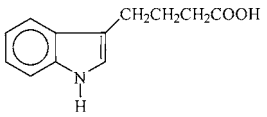
The aim of this work is to demonstrate the applicability of capillary zone electrophoresis in the analysis of a test mixture complex of charged phytohormones using UV diode array detection. This is the first report of the simultaneous analysis of the five phytohormones (see **Figure 1**) extensively used in an intense agricultural zone in southern Spain.

MATERIALS AND METHODS

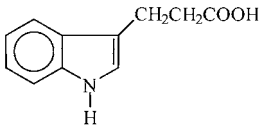
Reagents and Materials. All solutions were prepared with water purified by a Milli-Q^{uv} Plus system. Ammonium chloride was supplied by Panreac (Montcada I Reixac, Barcelona, Spain). Acetone and sodium phosphate were purchased from Merck (Darmstadt, Germany). Sodium dodecyl sulfate was from Sigma (St. Louis, MO), and sodium hydroxide was from Riedel-de-Häen (Seelze, Germany).

* Address correspondence to this author at the Department of Analytical Chemistry, Faculty of Sciences, University of Granada, C/Fuentenueva s/n, 18071 Granada, Spain (e-mail ansegura@ugr.es; fax +34-58-249510).

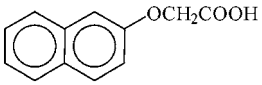
10.1021/jf0305377 CCC: $27.50
Published on Web 02/19/2004



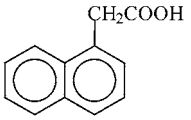
Indolebutyric acid (IBA)



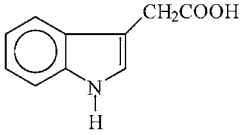
Indolepropionic acid (IPA)



Naphthoxyacetic acid (NOA)



Naphthalenacetic acid (NAA)



Indoleacetic acid (IAA)

Figure 1. Molecular structures for the five phytohormones studied.

The phytohormones indolebutyric acid (IBA), indolepropionic acid (IPA), indoleacetic acid (IAA), naphthoxyacetic acid (NOA), and naphthaleneacetic acid (NAA) were purchased from Aldrich (Madrid, Spain) and used as such. Stock solutions of each analyte were prepared in acetone, at a concentration of 1000 mg L^{-1}. Working standard solutions were prepared by diluting the stock standard solutions with 30 mM sodium phosphate solution (pH 7.25). The background electrolyte was prepared by dissolving the required amount of the substance in Milli-Q water to obtain the indicated final concentration.

Apparatus. Experiments were carried out on a Beckman P/ACE System MDQ capillary electrophoresis instrument. The system comprised a 0−30 kV high-voltage built-in power supply, equipped with a diode array UV−vis detector, and the P/ACE System MDQ software for system control and data handling. The voltage was applied through platinum electrodes. The separation capillary was made from bare fused silica obtained from Beckman Instrument, Inc. (Fullerton, CA) and had an internal diameter (i.d.) of 75 μm and a external diameter (e.d.) of 375 μm, with 57 cm of total length (L_T) with a 50 cm effective length (L_D) and a detector window of 100 × 800 μm.

Electrophoretic Conditions. The capillary was conditioned prior to its first use by flushing with 0.5 M NaOH for 10 min, followed with deionized water for 5 min, and finally with the background electrolyte solution for 5 min. This was important to maintain a consistent electrophoretic flow (EOF) from run to run because any variation results in poor migration time precision. To prevent difficulties owing to adsorption and to ensure a consistent EOF, the capillary was regenerated by rinsing with 0.5 M NaOH for 2 min, with ultrapure water for 2 min, and with fresh buffer for 2 min between runs.

Samples were injected by hydrodynamic injection for 10 s (50 nL). The electrolyte and operating potential were varied according to the experiments. Separations were carried out at a temperature of 25 °C with 30 kV (155 μA) and at a wavelength detection of 228 nm.

Sample Preparation. Samples of tomatoes were purchased at the local supermarket. A liquid−liquid extraction methodology (LLE) has been applied to extract the phytohormones. A representative portion of the vegetable samples was chopped and homogenized in a food processor (Ultra Turrax). Then, 150 g was weighed and placed into a centrifuge tube and homogenized with 300 mL of acetone by sonication for 30 s. Afterward, 300 mL of dichloromethane and 300 mL of petroleum ether were added to the mixture, and the homogenization was again carried out for 30 s. The resulting sample was centrifuged in a centrifuge tube for 2 min at 4000 rpm. The water-soluble layer was discarded, and the upper layer, corresponding to the organic extract, was decanted and evaporated to dryness. The dry residue was reconstituted in 2 mL of water.

Extraction recoveries were determined by fortifying fresh samples (150 g) with 0.01 mg of each analyte.

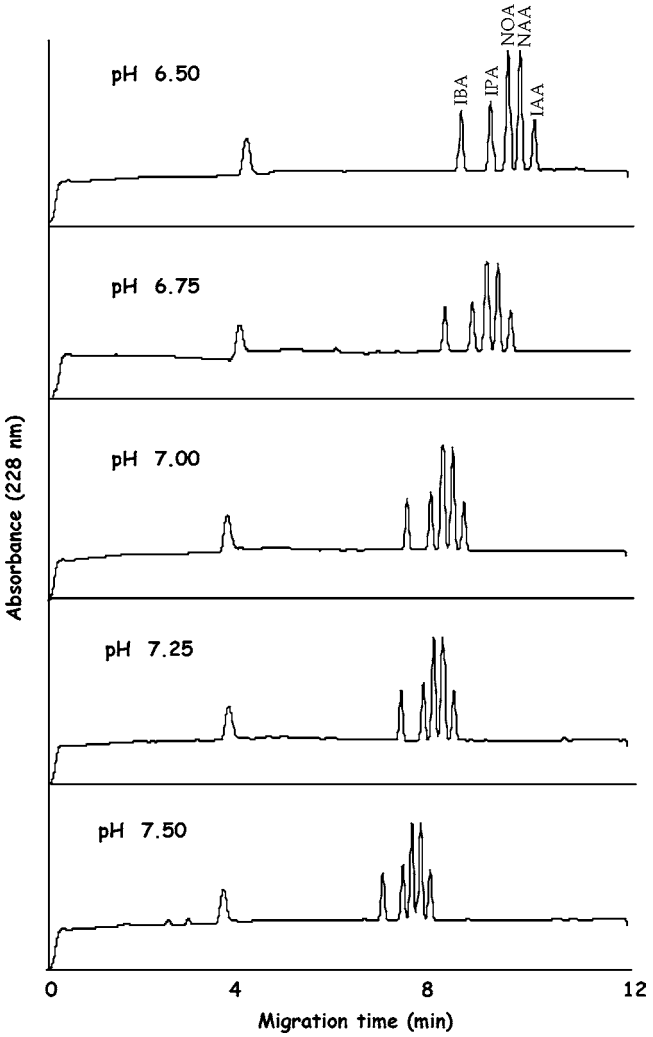


Figure 2. Electropherograms of the five phytohormones (5 mg L^{-1}) at different pH values. Operating conditions: 30 kV, 25 °C, 10 s injection. Experimental variables: 20 mM sodium phosphate buffer.

RESULTS AND DISCUSSION

The separation of the five charged phytohormones was carried out using the CZE technique. When CZE is employed, it is necessary to optimize the pH because it is important that the analytes are in their ionic forms. The influence of the pH using NaH_2PO_4/Na_2HPO_4 on the migration times and resolution of the analytes is presented in **Figure 2**.

Figure 3 shows the effect of the ionic strength of the run buffer on the migration times and peak heights of a standard mixture of the five phytohormones. The sample components were dissolved in water. When the run buffer concentration was increased from 10 to 30 mM, increases in peak efficiency and peak height were observed.

The difference in conductivity between the sample zone and the run buffer permits focusing (also referred to as sample

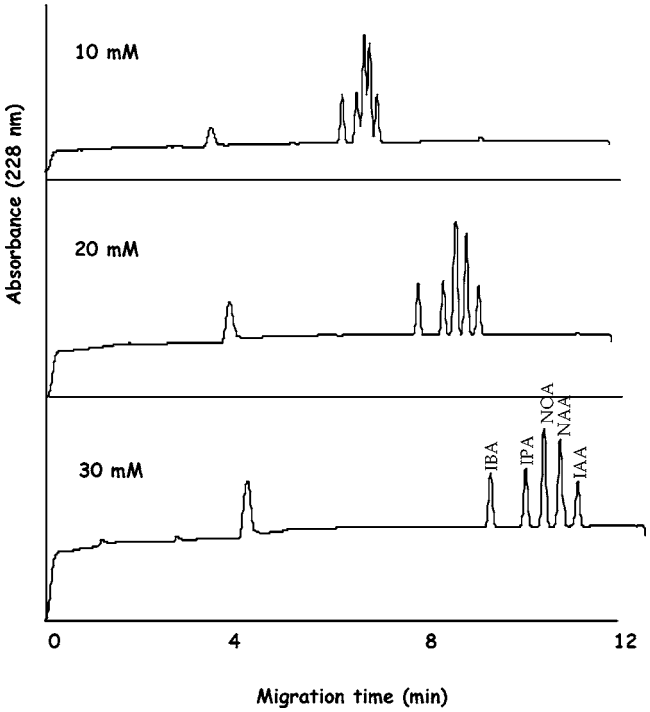


Figure 3. Electropherograms of the five phytohormones (5 mg L^{-1}) at different sodium phosphate buffer concentrations. Operating conditions: 30 kV, 25 °C, 10 s injection. Experimental variables: pH 7.25.

stacking). Under established conditions, a 5−10-fold increase in detection sensitivity can be obtained by using sample stacking. In this case, the electric field in the sample zone is relatively high, causing the analytes to migrate rapidly until they reach the interface between the sample buffer and the run buffer. This causes the sample to be "stacked" at that interface. Thus, the sample should be applied in a medium of relatively low conductivity. In the opposite case, uneven migration and zone spreading will result.

By dissolving analytes in water and using a pH 7.25 phosphate buffer, a 5−10-fold increase in signal can be obtained as compared to a sample matrix made of run buffer. The effect of salts dissolved in the sample matrix on peak shape is demonstrated in **Figure 4**. In this figure can be observed the good reproducibility in migration times of the phytohormones.

Calibration graphs were obtained by using liquid standards containing from 2 to 10 mg L^{-1} of the investigated phytohormones, to check the repeatability of the method. Each point of the calibration graph corresponds to the mean value obtained from three independent area measurements. These calibration graphs showed good correlation between peak areas of the phytohormone concentrations, with a regression coefficient of 0.99 in all cases.

The limits of detection (LOD) and quantification (LOQ) were calculated using the approach of Cuadros et al. (*29*) and were below 1.04 mg L^{-1} (0.014 mg kg^{-1}) for the different analytes under study. Calibration curves were studied by a series of injections of standard mixtures containing different concentrations of phytohormones. The different performance parameters for the five phytohormones under study are summarized in **Table 1**.

IBA, IPA, NOA, NAA, and IAA have been analyzed in vegetable samples of tomatoes. The CZE method was validated by a standard addition calibration methodology and applied to the determination of the five phytohormones in tomato samples.

Tomato samples were fortified at a 0.066 mg kg^{-1} level of each phytohormone by adding 10 μL of a solution of 1000 mg

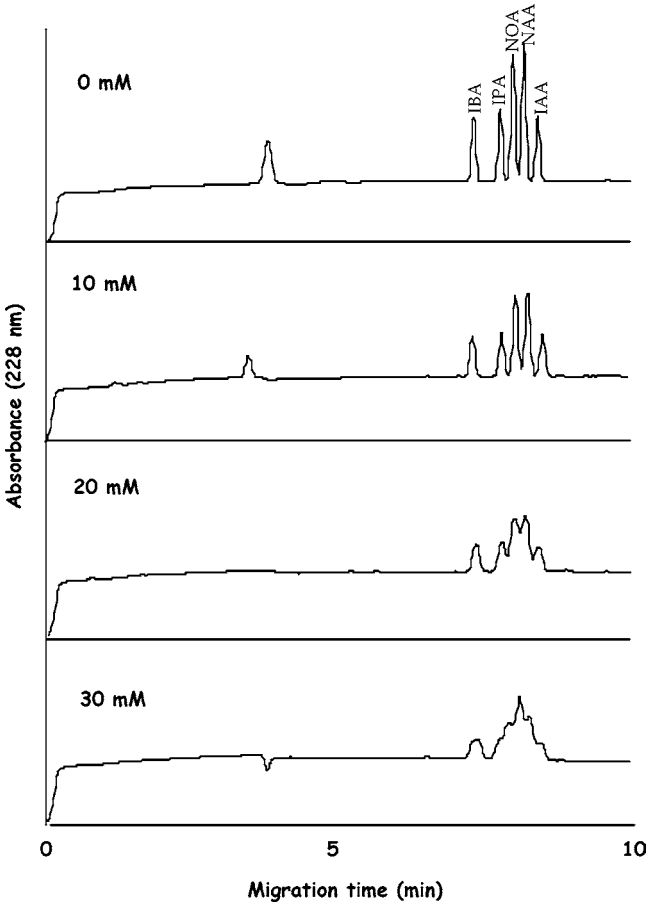


Figure 4. Electropherograms of the five phytohormones (5 mg L^{-1}) at different sodium phosphate concentrations in the sample. Operating conditions: 30 kV, 25 °C, 10 s injection. Experimental variables: pH 7.25 and 20 mM sodium phosphate buffer.

Table 1. Analytical Parameters of Proposed Method

	IBA	IPA	NOA	NAA	IAA
linear range (mg L^{-1})	2.3–10	1.48–10	2.86–10	3.46–10	1.99–10
detectn limit (mg L^{-1})	0.71	0.45	0.86	1.04	0.59
quantifn limit (mg L^{-1})	2.30	1.48	2.86	3.46	1.99
RSD (%)					
2 mg L^{-1}	11.6	6.8	13.4	16.3	9.3
4 mg L^{-1}	5.3	3.4	6.8	8.4	4.6
6 mg L^{-1}	3.0	2.0	3.7	4.4	2.6
8 mg L^{-1}	2.5	1.6	3.0	3.7	2.1
10 mg L^{-1}	2.3	1.4	2.7	3.3	1.9

Table 2. Recovery Values and Relative Standard Deviation (RSD) of Phytohormones in Tomato Samples

phytohormone	recovery (%)	RSD (%)
IBA	108	7.3
IPA	93	8.9
NOA	118	2.3
NAA	110	6.0
IAA	91	6.8

L^{-1} of each phytohormone over 150 g of tomato, and then the extraction procedure was applied. An electropherogram of a fortified tomato sample is shown in **Figure 5**. An electropherogram of the tomato sample before fortification was registered, but no peaks were obtained. The recovery values obtained for seven samples are given in **Table 2**.

These satisfactory results demonstrate the applicability of the CZE method to the separation and analysis of the five

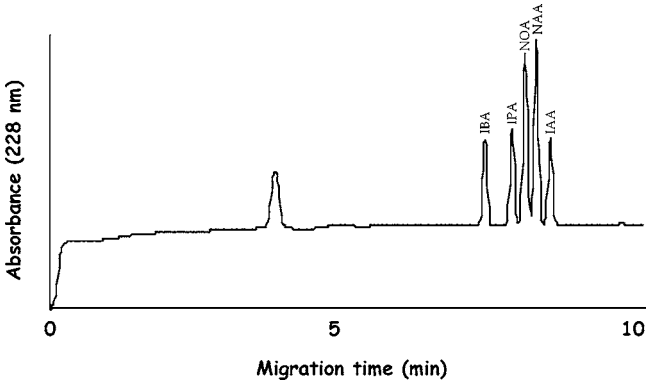


Figure 5. Electropherogram of tomato sample fortified with the five phytohormones. Experimental conditions: 30 kV, 25 °C, 10 s injection. Experimental variables: pH 7.25 and 20 mM sodium phosphate buffer.

phytohormones. The analysis time was found to be dependent on pH, buffer concentration, and applied voltage. The present method required only small sample sizes and small solvent volumes. It showed satisfactory recovery values, repeatability, and reproducibility, and it was sensitive and specific.

ACKNOWLEDGMENT

We greatly appreciate the collaboration with the research group Química Analítica de Contaminantes directed by Professor Jose Luis Martínez Vidal from Universidad de Almería.

LITERATURE CITED

(1) Deyl, Z.; Miksik, I.; Tagliaro, F. Advances in capillary electrophoresis. *Forensic Sci. Int.* **1998**, *92*, 89−124.
(2) Issaq, H. J. A decade of capillary electrophoresis. *Electrophoresis* **2000**, *21*, 1921−1939.
(3) Tagliaro, F.; Manetto, G.; Crivellente, F.; Smith, F. P. A brief introduction to capillary electrophoresis. *Forensic Sci. Int.* **1998**, *92*, 75−88.
(4) Valcárcel, M.; Arce, L.; Ríos, A. Coupling continuous separation techniques to capillary electrophoresis. *J. Chromatogr.* **2001**, *924*, 3−30.
(5) Jorgenson, J. W.; Lukacs, K. D. Zone electrophoresis in open-tubular glass-capillaries. *Anal. Chem.* **1981**, *53*, 1298−1302.
(6) Terabe, S.; Otsuka, K.; Ichikawa, K.; Tsuchiya, A.; Ando, T. Electrokinetic separations with micellar solutions and open-tubular capillaries. *Anal. Chem.* **1984**, *56*, 111−113.
(7) Corradini, C.; Cavazza, A. Application of capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) in food analysis. *Ital. J. Food Sci.* **1998**, *10* (4), 299−316.
(8) Issaq, H. J. Capillary electrophoresis of natural products. *Electrophoresis* **1997**, *18*, 2438−2452.
(9) Cruces-Blanco, C.; Segura-Carretero, A.; Gálvez Mata, L.; Fernández Gutiérrez, A. Simultaneous determination, by capillary zone electrophoresis, of multiple components of different industrial products. *Chromatographia* **2001**, *53* (7/8), 414−418.
(10) Tagliaro, F.; Smith, F. P.; Turrina, S.; Equisetto, V.; Marigo, M. Complementary use of capillary zone electrophoresis and micellar electrokinetic capillary chromatography for mutual confirmation of results in forensic drug analysis. *J. Chromatogr. A* **1996**, *735*, 227−235.
(11) Dinelli, G.; Vicari, A.; Bonetti, A. Separation of sulfonylurea metabolites in water by capillary electrophoresis. *J. Chromatogr. A* **1995**, *700*, 195−200.
(12) Pico, Y.; Rodríguez, R.; Manes, J. Capillary electrophoresis for the determination of pesticide residues. *Trends Anal. Chem.* **2003**, *22*, 133−151.
(13) Penmetsa, K. V.; Leidy, R. B.; Shea, D. Herbicide analysis by micellar electrokinetic capillary chromatography. *J. Chromatogr. A* **1996**, *745*, 201−208.
(14) Smith, J. T.; El Rassi, Z. Micellar electrokinetic capillary chromatography with in-situ charged micelles. 3. Evaluation of alkylglucoside surfactants as anionic butylboronate complexes. *Electrophoresis* **1994**, *15*, 1248−1259.
(15) Mechref, Y.; El Rassi, Z. Capillary electrophoresis of herbicides. 1. Precolumn derivatization of chiral and achiral phenoxy acid herbicides with a fluorescent tag for electrophoretic separation in the presence of cyclodextrins and micellar phases. *Anal. Chem.* **1996**, *68*, 1771−1777.
(16) Foret, F.; Sustácek, V.; Bocek, P. Separation of some triazine herbicides and their solvolytic products by capillary zone electrophoresis. *Electrophoresis* **1990**, *11*, 95−97.
(17) Schmitt, P.; Garrison, A. W.; Freitag, D.; Kettrup, A. Separation of s-triazine herbicides and their metabolites by capillary zone electrophoresis as a function of pH. *J. Chromatogr. A* **1996**, *723*, 169−177.
(18) Lanças, F. M.; Rissato, S. R.; Galhiane, M. S. Analysis of carbaryl and carbofuran in tobacco samples by HRGC, HPLC, and CZE. *J. High Resolut. Chromatogr.* **1996**, *19*, 200−206.
(19) Lanças, F. M.; Rissato, S. R.; Galhiane, M. S. Off-line SFE-CZE analysis of carbamates residues in tobacco samples. *Chromatographia* **1996**, *42*, 323−328.
(20) Jeong, M. L.; Chesney, D. J.; Determination of *N*-methylcarbamate pesticides from spiked matrices using supercritical fluid extraction. *Anal. Chim. Acta* **1999**, *389*, 53−57.
(21) Molina, M.; Wiedmer, S. K.; Jussila, M.; Silva, M.; Riekkola, M. L. Use of a partial filling technique and reverse migrating micelles in the study of *N*-methylcarbamate pesticides by micellar electrokinetic chromatography-electrospray ionization mass spectrometry. *J. Chromatogr. A* **2001**, *927*, 191−202.
(22) Sergeeva, S. I.; Chekurov, V. M. Using phytohormones as revealing hidden polymorphism factors in degree of lodging and drought-resistance of common wheat. In *Growth and Development Regulators of Plants*; Moscow, Russia, 1993; p 61.
(23) Chekurov, V. M.; Sergeeva, S. I. Using phytohormones in plant selection. In *Genetical Resources and Effective Methods of Creation of New Selective Agricultural Plant Material*; Novosibirsk, Russia 1994; p 97.
(24) Gaspar, T.; Kevers, C.; Faivre-Rampant, O.; Crevecoeur, M.; Penel, C.; Greppin, H.; Dommes, J. Changing concepts in plant hormone action. *In Vitro Cell. Dev. Biol. Plant* **2003**, *39*, 85−106.
(25) Khryanin, V. N. Role of phytohormones in sex differentiation in plants. *Russian J. Plant Physiol.* **2002**, *49*, 545−551.
(26) Terreaux, C.; Polasek, J.; Hostettmann, K. Plant constituents with hormonal effects. *Curr. Org. Chem.* **2003**, *7*, 1151−1161.
(27) Heikaus, S.; Winterhager, E.; Traub, O.; Grummer, R. Responsiveness of endometrial genes connexin26, connexin43, C• and clusterin to primary estrogen, selective estrogen receptor modulators, phyto- and xenoestrogens. *J. Mol. Endocrinol.* **2002**, *29*, 239−249.
(28) Soto, A. M.; Lin, T. M.; Justicia, H.; Silvia, R. M.; Sonnenschein, C. An "in culture" bioassay to assess the estrogenicity of xenobiotics (E-SCREEN). *J. Clean. Technol. E. T.* **1998**, *7*, 331−343.
(29) Cuadros Rodriguez, L.; García Campaña, A. M.; Jimenez Linares, C.; Román Ceba, M. Estimation of performance-characteristics of an analytical method using the data set of the calibration experiment. *Anal. Lett.* **1993**, *26*, 1243−1258.

Received for review July 29, 2003. Revised manuscript received December 16, 2003. Accepted December 19, 2003. We greatly appreciate the financial support of the Acciones Coordinadas de los Grupos de Investigación y Desarrollo Tecnológico de Andalucía and the Project of the Ministerio de Ciencia y Tecnología (BQU2002-03418) and Cajamar.

JF0305377