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Determination of capsaicin and dihydrocapsaicin by micellar electrokinetic capillary chromatography and its application to various species of *Capsicum,* Solanaceae

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Abstract

An easy, rapid and sensitive method of analysis for capsaicin and dihydrocapsaicin and its application for determination of these two amides in fruit extracts of different varieties of *Capsicum frutescens* by micellar electrokinetic capillary chromatography has been developed. Optimum separation was achieved with a fused-silica capillary column (600 mm×0.075 mm I.D) and a running buffer at pH 9.0 prepared from 15 m*M* sodium tetraborate and 15 m*M* sodium dihydrogenphosphate, and 67.5 m*M* sodium dodecyl sulphate. Addition of 15% (v/v) methanol in the running buffer was found to be essential for the separation. The applied voltage was +22.5 kV. The compounds were detected by UV at 214 nm. Both capsaicin and dihydrocapsaicin were detected within 11 min, with an excellent resolution.

Keywords: Fruits; Food analysis; Capsaicin; Dihydrocapsaicin; Amides

1. Introduction

Capsaicin (CAPS) and dihydrocapsaicin (DHC) are the main pungent components of hot chilli peppers (*Capsicum*, Solanaceae), constituting 90% or more of the total capsaicinoids [1,2]. The other 10% are nordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin. Several varieties of *Capsicum* are widely commercialized in many countries (mostly *Capsicum annuum* in temperate zones and *Capsicum frutescens* in tropical areas) [3]. In Réunion Island, a tropical French overseas department, these fruits are widely consumed and appreciated. Interest in CAPS is based on its toxicity (likely carcinogenic action) [4–7], its effects on the nervous system [8], and its nutritional and therapeutical benefits at low doses [8–11].

Previous chromatographic methods have been reported for analytical separation, quantitation and identification of naturally occurring capsaicinoids by gas chromatography [12–15], high-performance liquid chromatography [16–19] and thin-layer chromatography [20–23]. In 1986, Krajewska and Powers [24] described a reversed-phase low-pressure liquid chromatographic method for isolation of naturally occurring capsaicinoids. All these methods, though attaining sometimes low detection limits, are very laborious, long-lasting and expensive. The aim of our study was to find an easy, rapid, sensitive, and cheap method for the determination of CAPS and DHC. The influence of pH, the ionic strength of the buffer, the sodium dodecyl sulphate (SDS) concentration, the percentage of methanol in the buffer and the applied voltage on the separation is discussed.

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2. Experimental

2.1. Materials

CAPS (8-methyl-N-vanillyl-6-nonenamide), purity degree 98% and DHC (8-methyl-N-vanillyl-nonanamide), purity degree 90%, were obtained from Sigma (Sigma–Aldrich, Saint Quentin-Fallavier, France). SDS, $NaH_2PO_4 \cdot H_2O$ and $Na_2B_4O_7 \cdot 10H_2O$ were purchased from Aldrich (Sigma–Aldrich) and were of analytical grade. Reversed-phased chromatography Sep-Pak Classic C_{18} cartridges, short body, were obtained from Waters (Waters, Saint Quentin-Yvelines, France).

2.2. Equipment

A Quanta 4000 high-performance capillary electrophoresis system equipped with a positive power supply (Waters, Milford, MA, USA) was used for CE studies. Electropherograms were recorded with Maxima 820 Data, Management and Control Software equipped with a WD24 Chromatograph interface board (Waters). The analyses were performed with a 600 mm×0.075 mm I.D. fused-silica capillary column with an effective length of 525 mm to the detector. The polyimide coating at the detector window was removed by flaming followed by methanol wash. The capillary was conditioned on a daily basis by pressuring with 0.5 *M* potassium hydroxide for 10 min, ultrapure water for 10 min, and the running buffer for 10 min. Detection of both capsaicin and dihydrocapsaicin was accomplished at 214 nm. The operating voltage was +22.5 kV and driving current was approximately 0.120 mA. The solutions were loaded hydrostatically for 25 s. A 2 min purge was performed after each sample with the running buffer solution. The pH of the running buffer was adjusted with a Radiometer Analytical pH meter. A basic Sartorius scales for masses above 10 g and an analytical Labosi AA-160 (OSI, Maurepas, France) for smaller and more precise masses were used. Ultrapure, type I, reagent grade water was obtained by a Milli-Q plus purification system (Millipore, USA).

2.3. Buffer solution preparation

A phosphate–borate buffer, described by Morin and Dreux [25], was slightly modified. Optimal conditions for the separation were obtained by the buffer described hereunder. It was prepared by mixing an appropriate volume of 15 m*M* sodium tetraborate solution with a 15 m*M* sodium dihydrogenphosphate solution in order to obtain a value of pH 9.0. When the pH was adjusted, SDS was added to the solution, so that a final 67.5 m*M* SDS concentration could be obtained. Finally, methanol was added in order to obtain 15% (v/v) in methanol. All buffers were degassed before use. The results obtained with this buffer for a standard solution of CAPS and DHC are displayed in Fig. 1.

2.4. Fruit extracts

Fresh fruits of each *Capsicum frutescens* variety were bought early in the morning at local farmers' markets, rapidly taken to our laboratory where they were washed and wiped thoroughly. Approximately 100 g were dried in an oven at 60°C overnight in order to determine the dry mass weight. Another 100 g were ground with the same quantity of methanol. Subsequently, the mixture was passed through a gaze and then filtered and degassed under vacuum in a Schleicher and Schuell apparatus with a 0.22 μm membrane filter. As soon as the filtrate came down, 1 ml of each sample was passed through a Waters Sep-Pak Classic C_{18} cartridge, short body, which performs reversed-phased chromatography by using a gradient of strongly to weakly polar solvents with nonpolar C_{18}. All unwanted hydrophilic species passed through the cartridge when the hydrophobic components were retained. The cartridge was washed with 10 ml of water (strongly polar solvent), and then hydrophobic species were eluted with 1.25 ml of methanol (less polar solvent). Approximately 95% of these substances were capsaicinoids among which were the two amides of interest. A 0.4 ml volume of this eluate were taken in a 0.5 ml Eppendorf, then immediately analysed in CE or stored in the freezer. When necessary, appropriate dilutions were carried out in order to match within the standard solution's calibration range (for CAPS: $5.112 \cdot 10^{-4}$ AU to

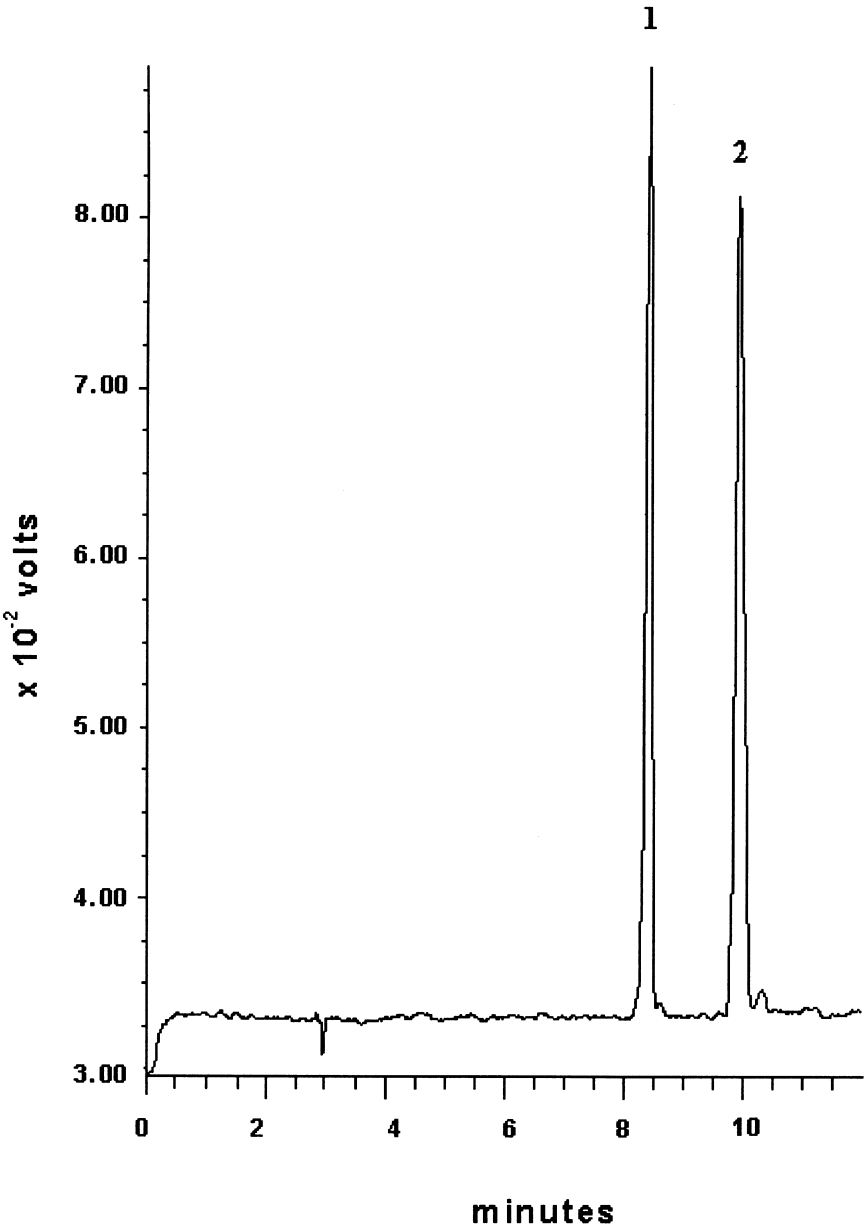

Fig. 1. Electropherogram of a standard solution of CAPS (1) and DHC (2). Running buffer: $Na_2B_4O_7 \cdot 10H_2O$–$NaH_2PO_4 \cdot H_2O$ buffer prepared from 15 m*M* sodium tetraborate and 15 m*M* sodium dihydrogenphosphate (pH 9.0) and 67.5 m*M* SDS with final 15% (v/v) methanol; column: fused-silica capillary column (600 mm×0.075 mm I.D); injection: hydrostatic mode for 25 s; voltage: +22.5 kV; direct detection at UV 214 nm.

$9.551 \cdot 10^{-2}$ AU and for DHC: $9.289 \cdot 10^{-4}$ AU to $7.827 \cdot 10^{-2}$ AU). When this procedure was used for fruit extracts, a standard solution underwent the same treatment on another identical cartridge so that eventual losses could be calculated. Ninety-eight % of the standard solution were recovered.

2.5. Standard solution preparation

Solutions of 2.458 m*M* CAPS and 2.978 m*M* DHC were prepared daily in a 50% (v/v) water and 50% methanol solution. Appropriate dilutions of this initial solution were prepared in order to obtain the calibration curves.

3. Results and discussion

Optimal conditions were determined for the standard solution. We have to underline that in our standard solutions, two minor peaks, one just after CAPS and the other just after DHC appeared under some experimental conditions. We think that these peaks correspond to the impurities contained in our commercial CAPS (90% purity) and DHC (98% purity) and represent minor capsaicinoids such as nordihydrocapsaicin, homocapsaicin or homodihydrocapsaicin (Fig. 1). Unfortunately, to our knowledge, none of these is commercially available, so that we may determine optimal conditions for a satisfactory separation of all these capsaicinoids. Thus, the choice of our optimal experimental conditions was guided by the presence of these impurities. When we subsequently checked their application to the eluate of our fruit extracts, we obtained the two amides of interest as well as other capsaicinoids—among small quantities of other hydrophobic species.

3.1. The running buffer concentration effect on the separation

One of the two parameters with the greatest influence on the resolution of the amides of interest was the running buffer concentration. Various concentrations of the running buffer were prepared (from 2.5 to 27.5 m*M*) and their effect on the resolution were tested (driving current varied from 0.050 to 0.260 mA). The more the buffer concentration increased, the more the migration time decreased. The best resolution was obtained with a 15 m*M* buffer (Fig. 2). Interesting resolutions were also obtained from 20 to 27.5 m*M* but increasing the ionic strength, also increases the current at the constant voltage of 22.5 kV, disabling the analysis

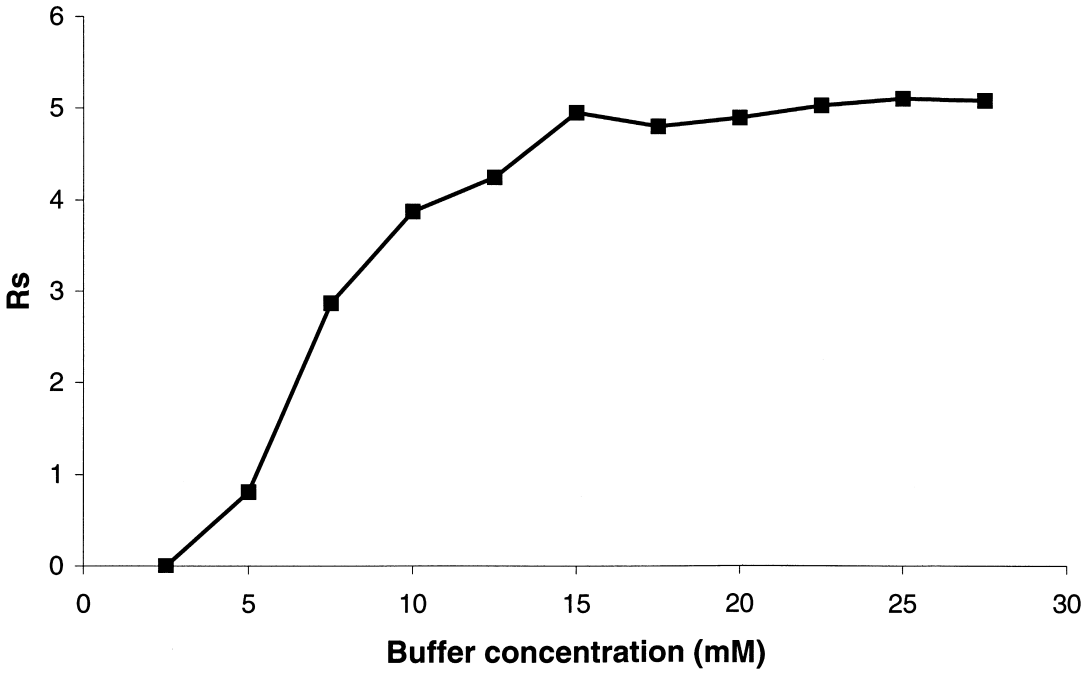

Fig. 2. Effect of buffer concentration (2.5–30 m*M*) on resolution (R_s) of CAPS and DHC. Other conditions as in Fig. 1.

most of the time. It was impossible to run the analysis over the buffer concentration value of 27.5 m*M*. Thus, the 15 m*M* buffer concentration was chosen for optimal conditions.

3.2. The running buffer pH effect on the separation

Various pH (from 5.5 to 9.5) of the running buffer were prepared and their effect on the separation was checked. The driving current varied from 0.100 to 0.160 mA. Lower pH did not achieve a good separation of CAPS and DHC, as shown in Fig. 3. We obtained an optimum resolution for a pH value of 9.0. A decrease in the migration time was observed, as the pH increased.

3.3. SDS concentration influence

Different concentrations of SDS (30–105 m*M*) were tested (driving current varied from 0.070 to 0.20 mA). The more the buffer concentration increased, the more the migration time decreased. The best resolution was obtained for the value of 67.5 m*M* SDS concentration. Interesting resolutions were also obtained from values of 82.5 to 105 m*M* SDS but most of the time high ionic strength disabled the analyses. Thus, the 67.5 m*M* SDS concentration was chosen for optimal conditions (Fig. 4).

3.4. Methanol concentration influence

The parameter with the greatest influence on the separation was the methanol percentage (v/v) in the running buffer. Adding various percentages of methanol (0–20%) to the running buffer resulted in separation improvement of CAPS and DHC (driving current varied from 0.165 to 0.100 mA). The improvement of the resolution was almost linear with the increase of methanol percentage. But, as it has been shown previously by other authors, longer migration times were observed, together with peak broadening above 15% of methanol (Fig. 5). Thus, a compromise of good separation and short migration times, obtained with 15% methanol, was chosen for optimal conditions.

3.5. Applied voltage influence on resolution

The effect of various values of applied voltage (20–25 kV with driving current varying from 0.080 to 0.165 mA) was checked on the resolution of the two amides. Perfect linearity was obtained in the plot of observed current vs. applied voltage for the values

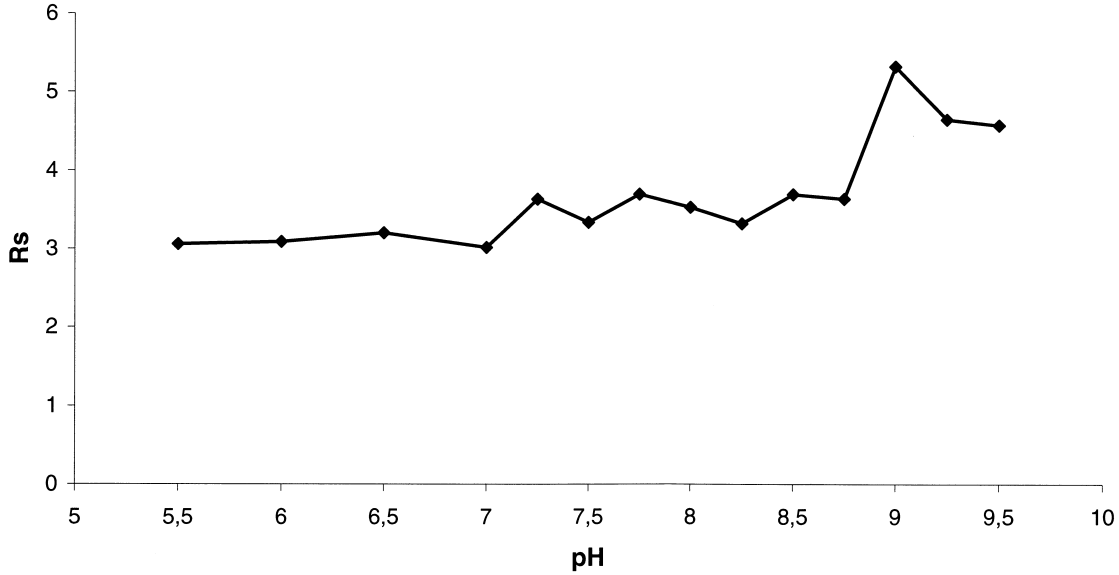

Fig. 3. Effect of pH (5.5–9.5) on resolution (R_s) of CAPS and DHC. Other conditions as in Fig. 1.

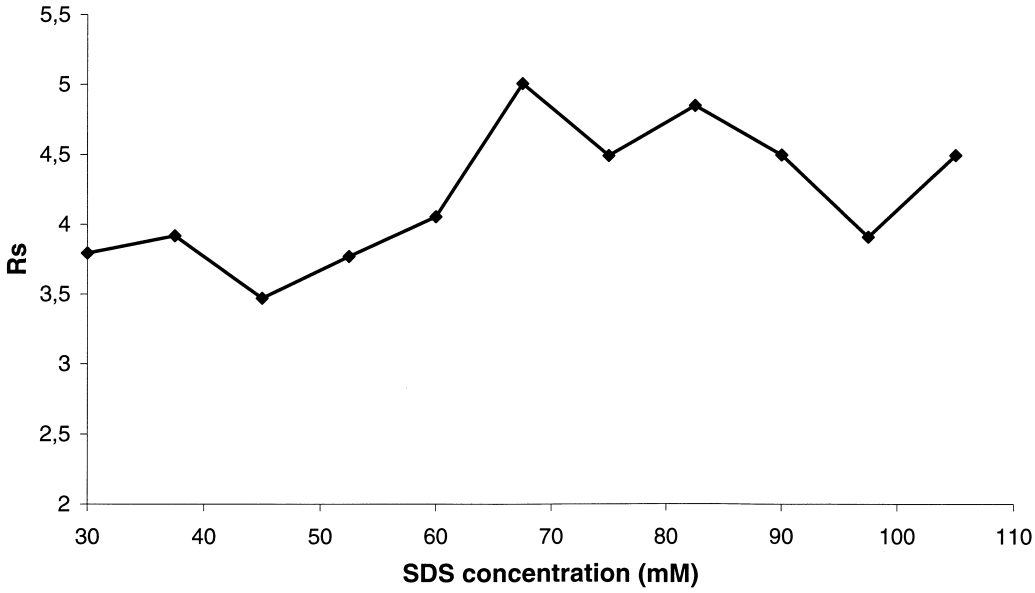

Fig. 4. Effect of SDS concentration (30–105 m*M*) on resolution (R_s) of CAPS and DHC. Other conditions as in Fig. 1.

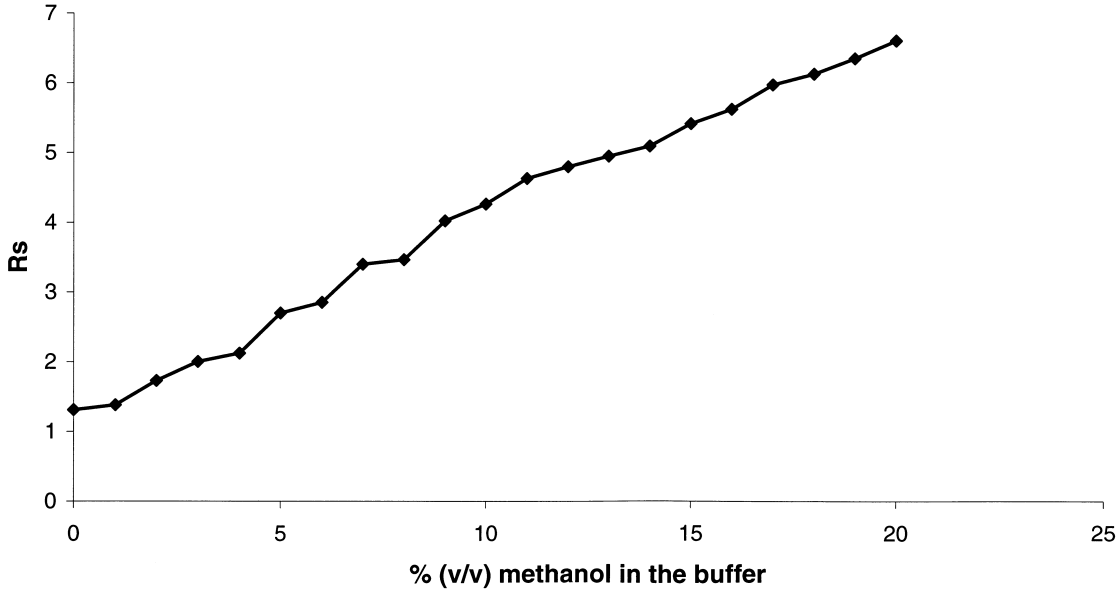

Fig. 5. Effect of methanol percentage (v/v) in the buffer (0–20%) on resolution (R_s) of CAPS and DHC. Other conditions as in Fig. 1.

between 20–23.5 kV (Ohm's law plot), as described by Nelson et al., [26] indicating that the generated Joule heat was effectively dissipated for these values. Linearity was lost over the value of 23.5 kV, reflecting the increase in capillary temperature. As expected, increasing the applied voltage resulted in

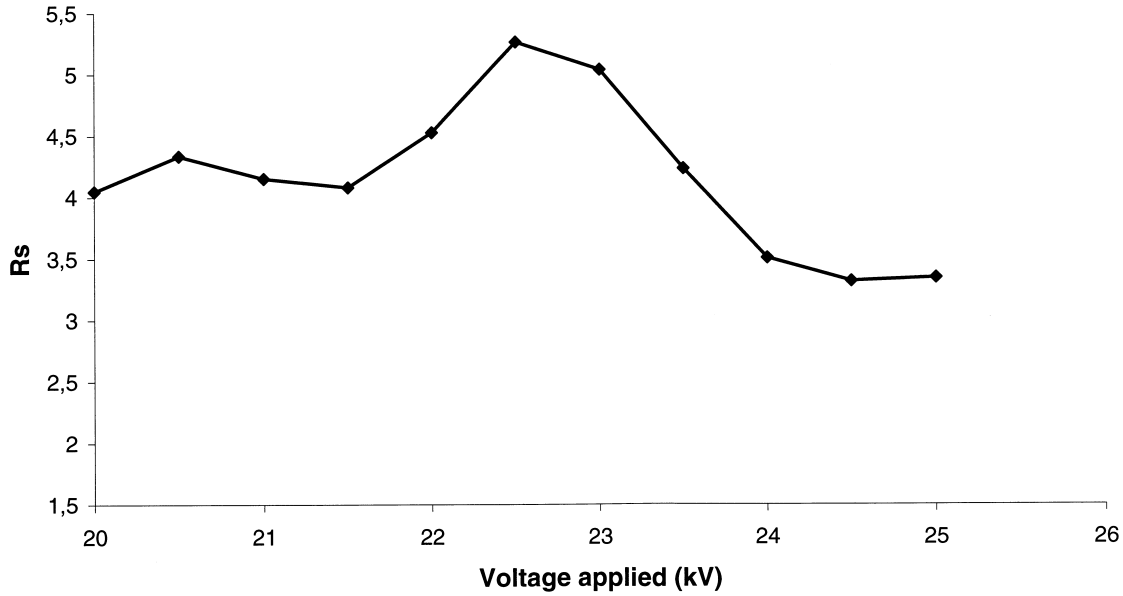

Fig. 6. Effect of voltage (20–25 kV) on resolution (R_s) of CAPS and DHC. Other conditions as in Fig. 1.

shortening migration time. The resolution was optimal for a value of 22.5 kV (Fig. 6).

3.6. Calibration

Determination of the two amides in the fruit extracts was performed by using the external standard method. For quantification purposes, the raw peak area was divided by the corresponding migration time (corrected area). The calibration graphs were expressed as corrected area vs. concentration in the concentration range. Excellent linearity was obtained for both CAPS and DHC in the range studied (1.755–750.6 mg/l for CAPS and 2.14–915.32 mg/l for DHC) with correlation coefficients for both equal to 0.999, as shown in Table 1. The detection limit was approximately 2 mg/l for both. For actual analyses, the system was calibrated daily.

3.7. Reproducibility

Reproducibility was evaluated by 20 consecutive runs with both CAPS and DHC in a standard solution. The relative standard deviations (R.S.D.) of migration time for CAPS and DHC were excellent (R.S.D. 1.078% for CAPS and 0.546% for DHC). The instrument repeatability data for the corrected area calculation for a standard solution of a con-

Table 1
Calibration range, regression equation, correlation coefficient, minimum detection limit and signal-to-noise ratio for capsaicin and dihydrocapsaicin

	Calibration range (mg/l)	Regression equation	Correlation coefficient	Minimum detection limit (mg/l)	Signal-to-noise ratio
CAPS	1.75–750.6	$y=-5.2+0.009054x$	$r=0.999$	1.75	3
DHC	2.14–915.3	$y=-8.6+0.01253x$	$r=0.999$	2.3	3

Experimental conditions as in Fig. 1.

Table 2
Reproducibility of migration time and calculated concentration for CAPS and DHC (n=20)

	Mean of migration time (min)	R.S.D. of migration time (%)	Mean of calculated concentration (mg/l)	R.S.D. of calculated concentration (%)
CAPS	8.304	1.078	572.55	2.18
DHC	9.862	0.546	555.77	1.72

Experimental conditions as in Fig. 1.

centration of 572.55 mg/l for CAPS and 555.77 mg/l for DHC were satisfactory (R.S.D. 2.18% for CAPS and 1.72% for DHC). These results are displayed in Table 2.

3.8. Determination of CAPS and DHC in Capsicum extracts

We chose to partially purify our crude extracts with the Waters reversed-phased chromatography Sep-Pak C_{18} cartridge rather than the classical methods such as Soxhlet extraction, because we wanted to accomplish a procedure which is rapid and uses the fewest organic solvents possible. The results have been more than satisfactory as the major peaks we obtained after the elution with methanol corresponded to our two capsaicinoids. The loss of CAPS and DHC, calculated with a standard solution, quantified before and after the cartridge was less than 2%.

Unfortunately, it was not possible to obtain the exact botanical identification of the *Capsicum frutescens* varieties, so we will use their local names. The mean mass of one pepper for each variety, the number of analyses, the contents of CAPS and DHC in three different varieties, as well as standard deviations are shown in Table 3. The results obtained correspond very well to different degrees of the burning taste obtained by each one of these *Capsicum*. The electropherograms are displayed in Fig. 7, (a) for ''gros piment'' which has a moderate burning taste, (b) for ''petit piment'' which has a strong burning taste and (c) for ''piment doux'' which has only a slight burning taste.

4. Conclusion

Micellar electrokinetic capillary chromatography has been developed for the qualitative and quantitative determination of CAPS and DHC from three *Capsicum frutescens* varieties. The two amides separated well within 11 min, with good sensitivity as well as linearity of the method. DHC was found to be more sensitive than CAPS. The analysis of the two amides in *Capsicum* extracts was possible with a simple grinding with methanol and a passage on a Waters reversed-phased chromatography Sep-Pak C_{18} cartridge. This method was found to be easy, rapid and cheap. Further investigations concerning other capsaicinoids will be conducted.

Table 3
Various *Capsicum frutescens* varieties with their local common names, their contents of CAPS and DHC, number of analyses, standard deviations and mean weight of one pepper

	CAPS Number of analyses (mg/100 g fruit)±standard deviation	DHC Number of analyses (mg/100 g fruit)±standard deviation
''Gros piment''	10	10
(big hot chilli pepper)		
Mean weight of one pepper: 10.6 g	22.22±2.12	150.70±10.63
''Petit piment''	10	10
(small hot chilli pepper)		
Mean weight of one pepper: 0.55 g	176.29±16.86	78.72±7.36
''Piment doux''	10	10
(mild hot chilli pepper)		
Mean weight of one pepper: 45 g	10.046±0.909	0

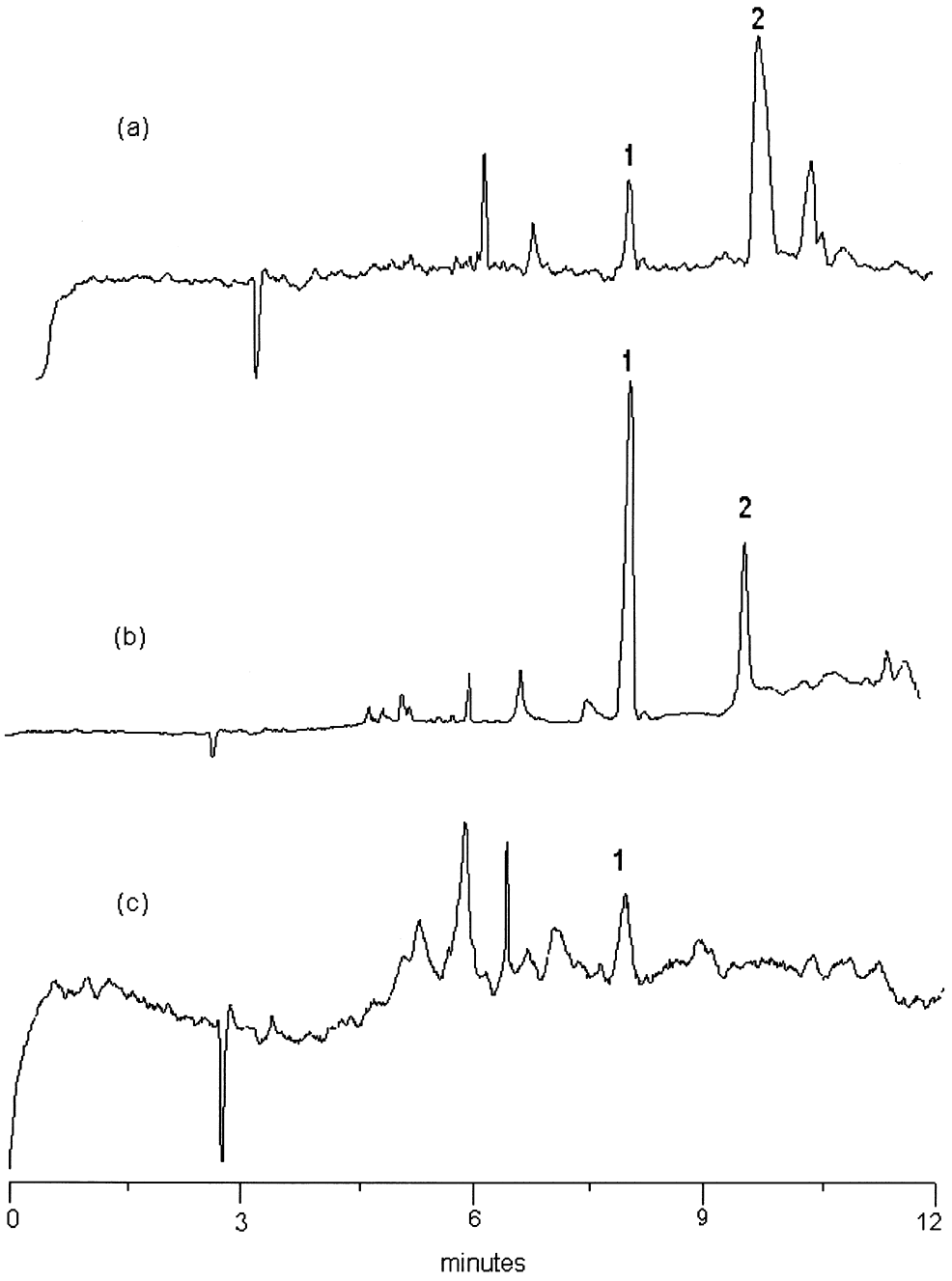

Fig. 7. Electropherograms of *Capsicum* varieties: (a) for ''gros piment'', (b) for ''petit piment'' and (c) for ''piment doux''. Peaks: 1=CAPS; 2=DHC; other peaks unidentified. Experimental conditions as in Fig. 1.

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References

[1] K. Iwai, T. Suzuki, H. Fujiwake, Agric. Biol. Chem. 43 (1979) 2493.
[2] A. Müller-Stock, R.K. Joshi, J. Büchi, J. Chromatogr. 63 (1971) 281.
[3] R. Lavergne, in: H. Cazal (Ed.), Fleurs de Bourbon, Vol. 6, Réunion Island, 1981, p. 133.
[4] B. Toth, E. Rogan, B. Walker, Anticancer Res. 4 (1984) 117.
[5] M. Nagabhushan, S.V. Bhide, Environ. Mutagen. 7 (1985) 881.
[6] T. Lawson, P. Gannett, Cancer Lett. 48 (1989) 109.
[7] L. Lopez-Carrillo, M.H. Avila, R. Dubrow, Am. J. Epidemiol. 139 (1994) 263.
[8] R. Gamse, A. Wax, R.E. Zigmond, S.E. Leeman, Neuroscience 6 (1981) 437.
[9] J.S. Lacroix, J.M. Buvelot, B.S. Polla, J.M. Lundberg, Clin. Exp. Allergy 21 (1991) 595.
[10] L. Lundblad, E. Brodin, J.M. Lundberg, A. Anggard, Acta Oto-Laryngol. (Stockholm) 100 (1985) 117.
[11] R.E. Papka, J.B. Furness, N.G. Della, M. Costa, Neurosci. Lett. 27 (1981) 47.
[12] Y. Masada, K. Hashimito, T. Inoue, M. Suzuki, J. Food Sci. 36 (1971) 858.
[13] P.H. Todd, M. Bensinger, T. Biftu, J. Food Sci. 42 (1977) 660.
[14] K. Iwai, T. Suzuki, H. Fujiwake, S. Oka, J. Chromatogr. 172 (1979) 303.
[15] J. Jurenitsch, R. Leinmüller, J. Chromatogr. 189 (1980) 389.
[16] O. Sticher, F. Soldati, R.K. Joshi, J. Chromatogr. 166 (1978) 221.
[17] J. Jurenitsch, E. Bingler, H. Becker, W. Kubelka, Planta Med. 36 (1979) 54.
[18] F. Heresh, J. Jurenitsch, Chromatographia 12 (1979) 647.
[19] J. Jurenitsch, I. Kampelmühler, J. Chromatogr. 193 (1980) 101.
[20] S. Kosuge, M. Furuta, Agric. Biol. Chem. 34 (1970) 248.
[21] P.H. Todd, M. Bensiger, T. Biftu, J. Chromatogr. Sci. 13 (1975) 577.
[22] T. Suzuki, T. Kawada, K. Iwai, J. Chromatogr. 198 (1980) 217.
[23] D.S. Pankar, N.G. Magar, J. Chromatogr. 144 (1977) 149.
[24] A.M. Krajewska, J.J. Powers, J. Chromatogr. 367 (1986) 267.
[25] Ph. Morin, M. Dreux, J. Liq. Chromatogr. 16 (1993) 3735.
[26] R.J. Nelson, A. Paulus, A.S. Cohen, A. Guttman, B.L. Karger, J. Chromatogr. 480 (1989) 111.