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Derivatization of dipeptides with 4-fluoro-7-nitro-2,1,3-benzoxadiazole for laser-induced fluorescence and separation by micellar electrokinetic chromatography

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Abstract

In capillary electrophoresis generally very small sample volumes are introduced, which often gives problems regarding determinations of low concentrations of the analytes. Frequently, therefore, they have to be transformed into products by suitable derivatization reagents. In this study 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was used as a pre-capillary fluorigenic reagent for a series of dipeptides used as model compounds in order to study the characteristics of the derivatization procedure. Main emphasis was put on optimization of the reaction conditions using a chemometric approach involving a fractional factorial design for screening experiments and a central composite face-centred design for response surface modelling. The results showed that the reagent excess must be at least 70 times in order to get a linear response, the reaction mixture should consist of a phosphate buffer with low ionic strength (0.001) at pH 7 containing 15% of isopropanol. The presence of the micellar agent Brij 35 in the background electrolyte increased the fluorescence intensity of the analyte product at least 3 times, and the separation selectivity increased compared to using a neat buffer. Leu–Val, chosen as a model peptide for studies on quantitative determinations, could be determined at the level 10^{-7} *M* (2 fmol injected) with a quantitative recovery and a relative standard deviation of 2.4%. The limit of detection was $4 \cdot 10^{-9}$ *M* (70 amol injected).

1. Introduction

The determination of peptides present in complex matrices, like biological materials, in low concentrations requires generally derivatization procedures to be applied in combination with separation techniques like high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE). Many different reagents have been developed for the purpose, often being common for amino acids, peptides and proteins. The most used are probably *o*-phthalaldehyde (OPA) [1] and the related naphthalenedialdehyde [2]. For CE separations the derivatives are generally produced by off-line procedures [3–8], but post-capillary reactions have also been developed for OPA [9–12]. Post-capillary derivatization was advocated for the OPA reagent in studies on the analysis of some peptide-derived marine toxins due to degradation of the formed product [13]. Recently, OPA was utilized

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in a pre-column reaction coupled on-line to CE separations on a microchip [14]. Fluorescein-isothiocyanate (FITC) was introduced as a marker for the detection of proteins a long time ago [15], and has in recent years also been applied for the laser-induced fluorescence detection (LIF) of very low amounts of amino acids [16–19] and peptides [20] in CE separations. A reagent, 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA), designed to have spectral properties matching the output wavelength of the helium–cadmium laser (442 nm) and to give good migration behaviour of the products was presented by Novotny and co-workers [21]. The reagent was utilized in detection of small peptides [22] as well as larger ones [23] with cyclodextrin additives, and for the separation of amino acid homopolymers by capillary gel electrophoresis [24]. Other useful general fluorigenic reagents for peptide detection include fluorescamine [25,26] and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate [27]. In addition, reagents selective for arginine- [28,29] and tyrosine-containing [28] peptides have been presented.

For our studies an argon-ion laser with an output wavelength of 488 nm was available. Of the reagents discussed above only FITC and CBQCA have such spectral properties that they are potential candidates for use in combination with this laser. They were tested and compared with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), and the latter was found to be the best reagent for the dipeptides used as model compounds in this study. NBD-F was introduced by Imai and Watanabe in 1981 [30] and univariate studies indicated that a reaction for 5 min at pH 7.5 and 70°C was optimal for secondary amino acids. The reagent has been applied extensively in HPLC for the analysis of amines and amino acids (for a review see Ref. [31]).

The optimization of the reaction conditions for tagging dipeptides with NBD-F was performed by a chemometric approach applying a fractional factorial design followed by response surface modelling. The separations were performed at high pH by micellar electrokinetic chromatography (MEKC) with the non-ionic surfactant Brij 35 as additive.

2. Experimental

2.1. Apparatus

The instrument used in the experiments was a Beckman P/ACE system 2050 with a laser Model 488 and data were collected and analyzed using a Micro Scan 2E ADI computer (Beckman Instrument, Palo Alto, CA, USA). The argon-ion laser gives excitation at 488 nm, and the emission wavelength was 523 nm. The capillaries were Beckman fused-silica with 75 μm I.D., an effective length of 50 cm and a total length of 57 cm. The capillaries were thermostatted at 25°C. Before introduction of a new background electrolyte the capillaries were flushed with 0.1 *M* NaOH and water, then the capillaries were filled with new electrolyte and the system was allowed to equilibrate for about 10 min. The electrophoreses were carried out at a voltage between 9 and 15 kV. Samples were injected by pressure at 34 mbar (0.5 p.s.i.) for 5 s.

2.2. Chemicals

The background electrolyte consisted of boric acid p.A., phosphoric acid p.A. and sodium hydroxide p.A. from Merck (Darmstadt, Germany), diluted with deionized water. Methanol p.A. and isopropanol LiChrosolv were also from Merck. Ethanol (abs.) was from AB Kemetyl (Sweden).

The surfactant used was polyethylene glycol dodecyl ether (Brij 35) from FlukaBioChemie (Buchs, Switzerland).

The buffer solutions used in the reaction procedures were all prepared of chemicals from Merck and the quality was at least p.A.

The peptides were from Sigma (St. Louis, MO, USA), small amounts of CBQCA, FSE and NBD-F were gifts from Beckman; additional NBD-F was obtained from Molecular Probes (Eugene, OR, USA).

2.3. Reaction conditions

The reactions were initially performed according to guidelines from Beckman [32]: NBD-F in alcohol + the dipeptides in water + buffer pH 8

(2:2:1, v/v/v) were mixed, vortexed for 30 s, and the reaction was allowed to proceed for 20 min at 60°C. The concentration of alcohol in the mixture is then 40%. An aliquot of the reaction mixture was injected immediately after this period.

After the optimization studies the procedure was modified to give the highest yields: NBD-F (>70 times in excess) in isopropanol + the dipeptides in water + phosphate buffer pH 7 were mixed and vortexed for 30 s, followed by a reaction time of 50 min at 56°C. The final concentration of NBD-F must be $\geq 4 \cdot 10^{-4}$ *M*, and the volume of the reagent was chosen to give 15% of isopropanol in the final mixture. The ionic strength of the phosphate buffer was selected to give a ionic strength of 0.001 in the final mixture. Samples were then immediately injected for separation.

2.4. Optimal background electrolyte

As a result of optimization studies the following composition of the background electrolyte was chosen: borate–phosphate (25/12.5 m*M*) buffer, pH 9 ($I = 0.03$), containing 10 m*M* Brij 35. The use of a lower concentration of the micellar agent may in some cases be of advantage regarding selectivity.

2.5. Chemometric evaluation

The program MODDE from Umetri (Umeå, Sweden) was used for design and evaluation of the chemometric studies.

3. Results and discussion

3.1. Choice of reagent

In order to find a reagent suitable for use with the argon-ion laser available, with an output wavelength of 488 nm, four compounds were initially tested: FITC with an excitation wavelength maximum at 488 nm; CBQCA at 468 nm; 5-carboxyfluorescein succinimidyl ester (FSE), 491 nm; and NBD-F, 475 nm. Standard conditions recommended in the literature for an excess of the reagent [32] were used for the screening experiments, and Leu–Phe was selected as the model dipeptide. The results of this preliminary evaluation are given in Table 1.

With FITC the maximal fluorescence of the analyte was obtained after 4 h reaction, but many additional peaks originating from the reagent appeared in the electropherogram. It was concluded that a purification of the reagent or modified reaction conditions were necessary to develop FITC as a reagent useful for quantitative peptide assays. FITC has been frequently used as tag for amines, peptides and proteins giving derivatives with very intense fluorescence. However, according to our knowledge it has not been used for quantitative purposes; instead, a deficit of the reagent has been applied in the published reactions.

CBQCA was not tried extensively in the screening experiments, but the results indicated that the obtained derivative had a comparatively low fluorescence. One reason may be that the maximum excitation wavelength is about 20 nm away from the laser output. An advantage with

Table 1
Preliminary evaluation of reagents

	FITC	CBQCA	FSE	NBD-F
Exc. wavelength (nm)	488	468	491	475
Reaction time (h)	4	1	1	0.3
Reaction temp.	RT[a]	RT	RT	60°C
Response	n.d.[b]	low	high	high
Reagent peaks	many	none	many	few
Reagent fluorescence	high	none	high	high

[a] Room temperature.
[b] Not determined.

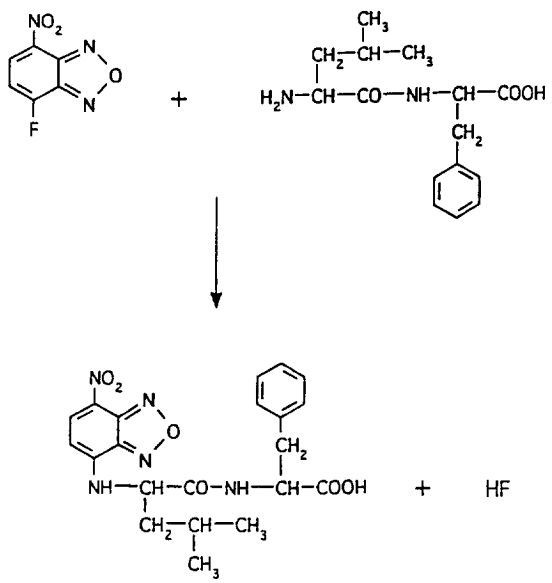

Fig. 1. Reaction of NBD-F with Leu–Phe.

CBQCA is a relatively fast reaction and that the reagent itself is non-fluorescent.

FSE with a maximum excitation wavelength close the laser light gave the highest response for the analyte product of all tested reagents, and the reaction time was relatively short (1 h). However, there were many additional potential interfering peaks in the electropherogram, indicating that an extensive clean up of the reagent is necessary before it can be applied for quantitative purposes.

NBD-F, finally, gave a good response for the analyte peak after a short reaction time (less than 1 h) and the electropherogram was reasonably clean. In addition, its cheaper chloride analogue (NBD-Cl) was tried as a reagent. However, a disadvantage of that is a longer reaction time, increasing the risk for side reactions. Consequently, NBD-F was chosen as the most suitable reagent for further development. The reaction of this reagent with Leu–Phe is illustrated in Fig. 1. The product has an intact carboxyl function which will be charged at the slightly alkaline conditions used both in the reaction and during the capillary electroseparation presented below.

3.2. Preliminary screening of reaction conditions

Reagent excess

The reaction conditions used in the literature typically involve an excess of the reagent in slightly alkaline (pH 7–10) solutions containing an alcohol. Studies on the required excess of reagent were performed in solutions containing methanol, ethanol and isopropanol with borate–phosphate or bicarbonate buffers. The reaction time was 30 min and the temperature 56°C. A representative result is shown in Table 2, applying the reagent dissolved in isopropanol mixed

Table 2
Dependence of reaction yield on reagent concentration and excess

Leu–Phe conc. (*M*)	Peak height (fluorescence) at NBD-F conc. (*M*)		
	$4.12 \cdot 10^{-5}$	$4.12 \cdot 10^{-4}$	$4.12 \cdot 10^{-3}$
$6 \cdot 10^{-8}$	–	0.7	–
$6 \cdot 10^{-7}$	0.36	7	–
$6 \cdot 10^{-6}$	-	71	114
$6 \cdot 10^{-5}$ (80% IPA)	–	30	–

Detection: LIF (488 nm). Capillary: Beckman fused-silica, 570 × 0.075 mm I.D., effective length: 500 mm. Background electrolyte: 10 m*M* Brij 35 in borate–phosphate buffer, pH 9. Voltage: 10 kV. Injection: 34 mbar for 5 s. Reagent mixture: NBD-F in isopropanol–Leu–Phe in water–$NaHCO_3$ 0.01 *M* (2:2:1) (40% isopropanol in the final mixture). Reaction time: 30 min. Reaction temperature: 56°C.

with 0.01 M $NaHCO_3$; the analyte (Leu–Phe) was generally added dissolved in water in different concentrations. The background electrolyte used in the capillary electroseparations was a borate–phosphate buffer, pH 9, containing 10 mM of the micellar agent Brij 35. A linear response of analyte product peak heights was obtained in the concentration interval $6 \cdot 10^{-8}$ to $6 \cdot 10^{-6}$ M using an NBD-F concentration of $4 \cdot 10^{-4}$ M. Increasing the reagent concentration ten times further gave an additional increase of the peak height. However, a too high reagent excess will increase the risk for the appearance of interfering peaks. On the other hand, decreasing the reagent concentration ten times gave a much lower response, even when keeping the reagent excess to ca. 70 times. Furthermore, when the analyte also was dissolved in the alcohol, giving a concentration of 80% in the reaction mixture, a considerably lower response was obtained. The result of the study indicates that the reagent must be added in a concentration $\geq 4 \cdot 10^{-4}$ M at an excess of at least 70 times, and the concentration of the alcohol must be kept adequately low.

Buffer

Studies performed at pH 8 using sodium bicarbonate, phosphate and phosphate–borate buffers indicated equivalency in response measured as peak heights in the electropherogram when either ethanol or isopropanol was the organic solvent. Bicarbonate gave higher response than phosphate–borate with methanol as the solvent; the levels were, however, much lower than when applying the other two solvents. The ionic strength of the buffer had a strong impact on the response; decreasing the buffer concentration ten times gave >3 times higher response for the model peptide (Leu–Phe). Phosphate buffer was selected for further studies, due to a better buffer capacity at pH ≤ 8 compared to bicarbonate.

Reaction time and type of alcohol

As mentioned above, the presence of methanol in the reaction mixture gave a lower response than with ethanol or isopropanol, the difference

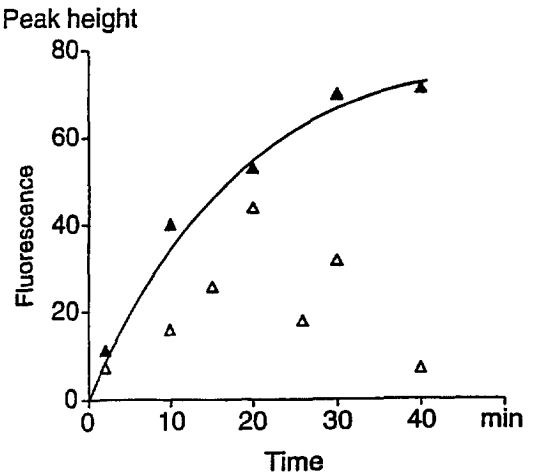

Fig. 2. Dependence of reaction yield on reaction time and type of alcohol (△ = ethanol; ▲ = isopropanol). Reaction conditions: NBD-F ($1 \cdot 10^{-3}$ M) in alcohol + Leu–Phe ($1.5 \cdot 10^{-5}$ M) in water + sodium bicarbonate (0.01 M) pH 8 (2:2:1, v/v/v) were mixed, vortexed for 30 s, and the reaction was allowed to proceed for 20 min at 60°C. Background electrolyte: borate–phosphate (25/12.5 mM) buffer, pH 9 ($I = 0.03$), containing 10 mM Brij 35.

was 2–3 times in peak height. A study on the reaction time was performed with ethanol and isopropanol, and the result is shown in Fig. 2. Reliable results were only obtained with isopropanol indicating that 30–40 min gave the maximal response. The data obtained using ethanol was severely scattered, and isopropanol was consequently chosen for further studies.

3.3. Conditions for capillary electrophoresis

A pH of 9–10 was chosen for the background electrolyte (BGE), mainly because the fluorescence intensity is maximal at high pH; in addition this will match the injected sample since the derivatization is performed at about the same pH, and it will also ensure complete ionization of the carboxylate group of the product, resulting in a high electrophoretic mobility. Scouting experiments showed that a borate–phosphate buffer at pH 9 gave much higher peak height, about 16 times, than a neat phosphate buffer at pH 10. It was also an advantage to use a low ionic strength of the buffer, i.e. applying the concentrations 25 and 12.5 mM of borate and phosphate gave a 1.4

times higher signal compared to twice the concentrations of the buffer components. It is well-known that fluorescence intensities may strongly depend on the environment, and the addition of the non-ionic micellar agent Brij 35 gave a further significant increase of the peak height, as illustrated in Fig. 3. The peak height of the analyte increased about 3 times when changing from neat buffer to a BGE containing 5 m*M* of Brij 35. Increasing the concentration of the micellar agent further gave additionally higher signals. This indicates that the reaction product gives a more intense fluorescence at more non-polar conditions. A similar increase was observed for the main reagent peak, which probably is the corresponding phenolic compound (–F is substituted by –OH), which at least partly is charged at the conditions used. The presence of the nitro group in *para*-position will increase the acidity of the phenol. The electroosmosis mi-

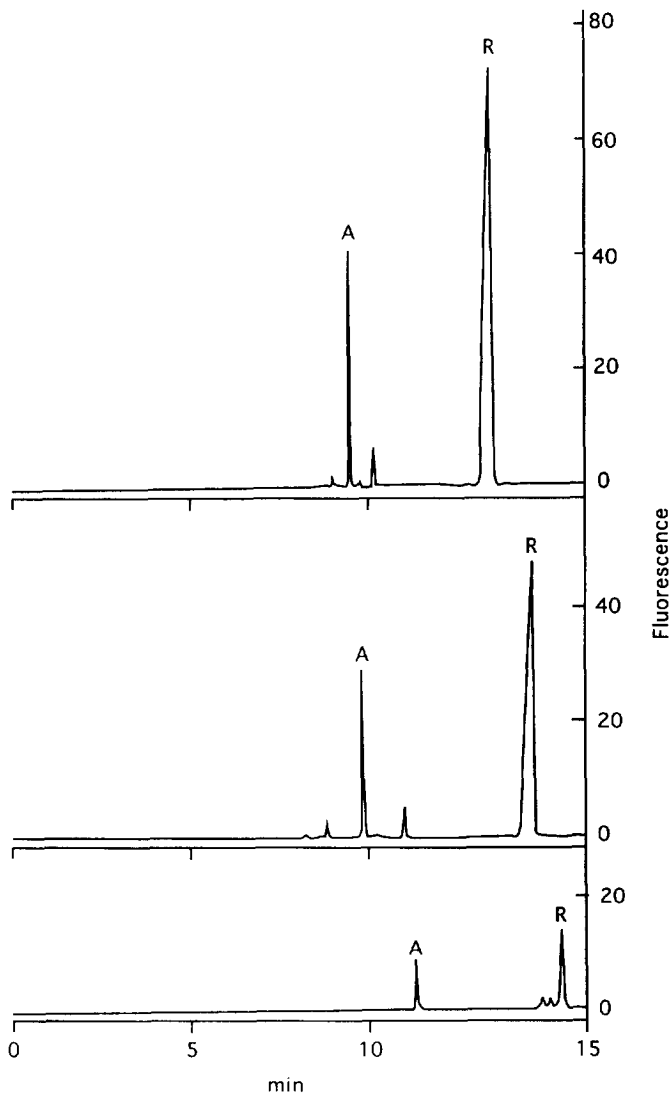

Fig. 3. Effects of Brij 35 in background electrolyte on fluorescence intensity. Background electrolyte: borate–phosphate buffer, pH 9 ($I = 0.03$), with or without Brij 35. The concentration of Brij 35 is increased from the bottom to the top electropherogram: 0, 5 and 10 m*M*, respectively. Voltage: 10 kV.

grates at 8–8.5 min in the different systems, corresponding to an apparent mobility of $(6–5.6)\cdot 10^{-4}$ $cm^2/V\cdot s$; the mobility decreasing somewhat with increasing micellar concentration. The micelles are non-ionic and migrate with the electroosmosis, and this means that the migration time of the analyte will decrease due to distribution to the micelles, since the analyte product is anionic and has an electrophoretic mobility towards the anode. The selectivity between the analyte and reagent products peaks increased in going from 0 to 5 m*M* Brij 35 (Table 3), and in addition some small impurity peaks became visible in the micellar system that were not observed in neat buffer. Some of those peaks may be hidden in the analyte peak when using plain buffer as the BGE. However, increasing the micellar concentration further, the selectivity decreases again, due to a too strong distribution of the components to the micellar phase. The efficiency is high in the systems (see Table 2) and increases with increasing micellar concentration from about 360 000 plates per m in the neat buffer system up to 540 000 with 10 m*M* Brij (the voltage was 10 kV).

3.4. Chemometric screening

Applying the experiences from the preliminary screening experiments described above, the most important variables were selected for a fractional factorial design experimental approach. It was decided to keep the reagent concentration constant at an excess of 100 times using the concentration $4\cdot 10^{-4}$ *M* in the derivatization of the model peptide Leu–Phe ($4\cdot 10^{-6}$ *M*). The variables studied were reaction time and temperature, ionic strength, pH and the concentration of isopropanol at the two levels given in Table 4. Sixteen experiments were performed according to the design also shown in the table. The peak areas were used as responses and they were plotted on a cumulative normal probability scale (*N*-plot), see Fig. 4. It was concluded that the three most important variables for the yield were the ionic strength, the pH and the amount of isopropanol, and the results indicated that lower values of all three factors than those chosen for this experimental series should be utilized. In addition there were some important interaction terms: temperature–ionic strength, temperature–pH and ionic strength–pH. Interestingly, the

Table 3
Efficiency and selectivity

Brij 35 conc. (m*M*)	Efficiency (*N*/m)	α [a] ($t_{m,R}/t_{m,A}$)
0	360 000	1.35
5	400 000	1.48
10	540 000	1.42

Detection: LIF. Capillary: Beckman fused-silica, 570 × 0.075 mm I.D., effective length: 500 mm. Background electrolyte: borate–phosphate buffer, pH 9, with or without Brij 35. Voltage: 10 kV. Current: 25 μA. Injection: 34 mbar for 5 s.
[a] $t_{m,R}$ = migration time of reagent; $t_{m,A}$ = migration time of analyte.

Table 4
Chemometric screening; fractional factorial design

Variable	Low level (−)	High level (+)
1. Time (min)	30	50
2. Temp. (°C)	56	76
3. Ionic strength	0.002	0.01
4. pH	8	9
5. Isopropanol (%)	20	40

Exp. no.	Variable				
	1	2	3	4	5
1	+	−	−	−	−
2	−	+	−	−	−
3	−	−	+	−	−
4	+	+	+	−	−
5	−	−	−	+	−
6	+	+	−	+	−
7	+	−	+	+	−
8	−	+	+	+	−
9	−	−	−	−	+
10	+	+	−	−	+
11	+	−	+	−	+
12	−	+	+	−	+
13	+	−	−	+	+
14	−	+	−	+	+
15	−	−	+	+	+
16	+	+	+	+	+

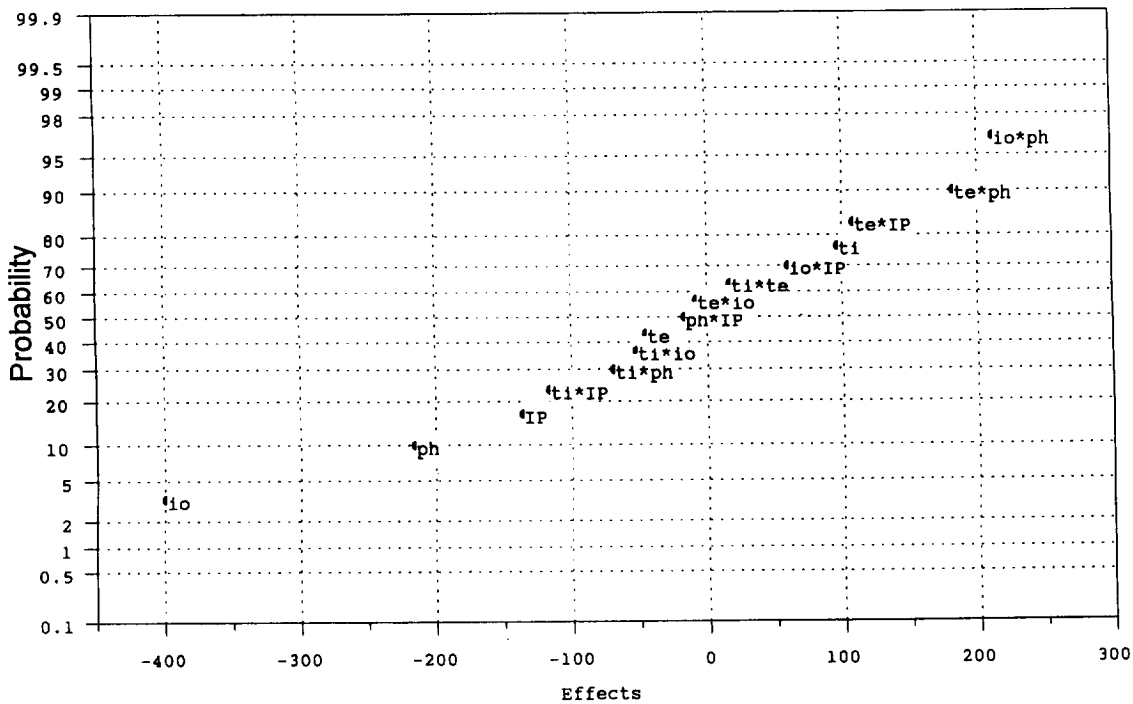

Fig. 4. *N*-plot of effects obtained after screening according to a fractional factorial design. Symbols: io = ionic strength; ph = pH; IP = isopropanol concentration; te = reaction temperature; ti = reaction time.

temperature itself had no significant effect on the response. There was also an indication that a longer reaction time had some impact on the outcome of the reaction. For further optimization of the reaction the three most important variables (ionic strength, pH and amount of isopropanol) were studied by response surface modelling, with the temperature and time kept constant at 56°C and 50 min, respectively.

3.5. Response surface modelling

A central composite face-centred (CCF) design was chosen for the response surface modelling (RSM) experiments. The ranges studied for the three variables were: ionic strength 0.001–0.002; pH 7.5–8; isopropanol 10–20%, and the experiments were run according to the worksheet given in Table 5. Four experiments (9, 10, 11 and 17) were unsuccessful according to the preliminary analysis by residuals plots and optimizations of the correlation between observed and predicted data, and were removed from the final analysis. The quadratic ionic-strength term gave a low significance according to an ANOVA analysis and was also eliminated. The multiple linear regression analysis gave an acceptable summary of fit with $R^2 = 0.982$, $R^2_{adj} = 0.953$ and $Q^2 = 0.710$, and a condition number of 4.683. The most important factors were the quadratic isopropanol term followed by the ionic strength and pH. The 3D contour plots (Fig. 5) show that there is an optimal isopropanol concentration (15%) and that the lowest values of the ionic strength and pH give the best response, indicating that even lower values would be optimal. However, it is not reasonable to use a lower ionic strength since this would give too low buffer capacity in the reaction, in which a strong acid (HF) is generated. The pH range chosen for the RSM studies was, however, too limited, and additional univariate experiments keeping the isopropanol amount to 15% and the ionic

Table 5
Worksheet for response surface modelling with a CCF design

ExpNo	ExpName	RunOrder	InOut	Ionic	pH	IPA	Area
1	N1	11	in	0.001	7.5	10	3049
2	N2	15	in	0.002	7.5	10	2983
3	N3	15	in	0.001	8	10	2757
4	N4	3	in	0.002	8	10	2583
5	N5	10	in	0.001	7.5	20	3125
6	N6	7	in	0.002	7.5	20	2854
7	N7	17	in	0.001	8	20	3103
8	N8	12	in	0.002	8	20	2589
9	N9	2	out	0.001	7.75	15	2596
10	N10	4	out	0.002	7.75	15	2563
11	N11	8	out	0.0015	7.5	15	2878
12	N12	6	in	0.0015	8	15	3100
13	N13	14	in	0.0015	7.75	10	2653
14	N14	5	in	0.0015	7.75	20	2843
15	N15	13	in	0.0015	7.75	15	3075
16	N16	9	in	0.0015	7.75	15	3029
17	N17	1	out	0.0015	7.75	15	1057
18	N18	16	in	0.0015	7.75	15	3066

Parameters: ionic strength (Ionic), pH, isopropanol conc. (IPA).

strength at 0.001 showed that pH 7 was optimal (pH 6.5 gave lower response).

3.6. Separation and quantitation

An illustration of the separation capability of the MEKC system is given in Fig. 6, where ten closely related dipeptides are separated after a simultaneous derivatization by the optimized reaction procedure. They are all migrating within a short time interval between 10 and 13 min, the reagent product peak does not interfere, since it migrates at 14.5 min.

A preliminary investigation of the potential of

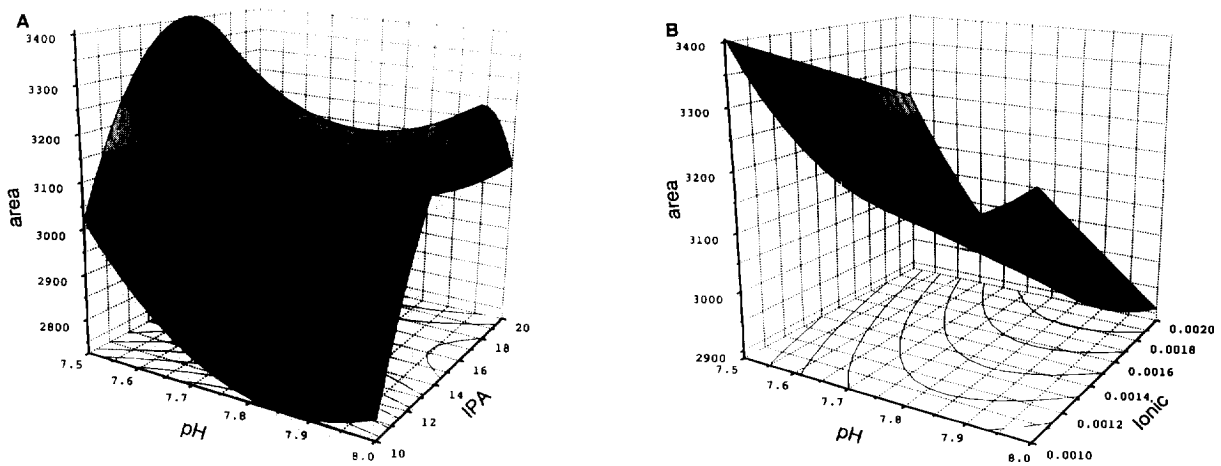

Fig. 5. Response surface modelling according to a central composite face-centred design: 3D contour plots. Experimental conditions: see Table 4. (A) Fixed parameter: ionic strength = 0.001. (B) Fixed parameter: isopropanol concentration = 15%.

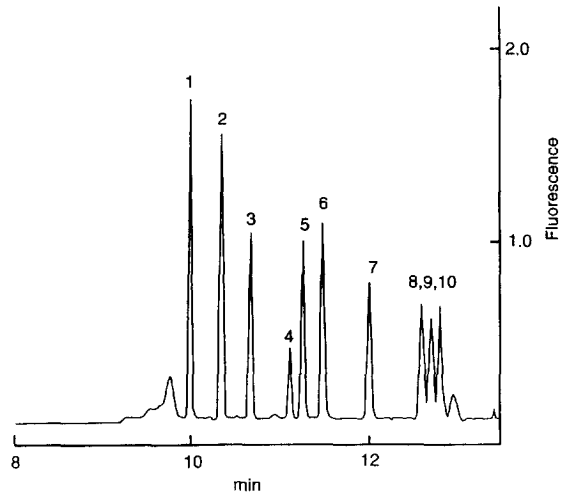

Fig. 6. MEKC separation of ten dipeptides. Reaction and capillary electrophoretic conditions according to the optimal procedures described in the Experimental section. Peptide concentrations: $4 \cdot 10^{-7}$ *M*. Peptides: 1 = Phe–Phe; 2 = Leu–Phe; 3 = Phe–Leu; 4 = Phe–Val; 5 = Leu–Leu; 6 = Leu–Val; 7 = Leu–Ala; 8 = Phe–Ser; 9 = Leu–Ser; 10 = Leu–Met. The reagent peak migrates at 14.5 min. Voltage: 9 kV; current: 20 μA.

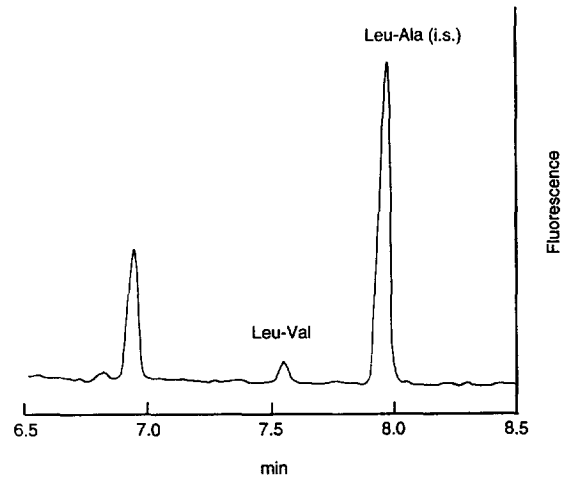

Fig. 7. Electropherogram from a quantitative analysis of Leu–Val. Reaction conditions: NBD-F ($4 \cdot 10^{-4}$ *M*) in phosphate buffer, pH 7 ($I = 0.001$), and isopropanol (13%); temperature 56°C; and reaction time 50 min. Background electrolyte: borate–phosphate buffer, pH 9, with 11 m*M* Brij 35. Leu–Val concentration: $5.2 \cdot 10^{-8}$ *M* (0.7 fmol injected). Leu–Ala concentration: $1.1 \cdot 10^{-7}$ *M*.

the procedure for quantitative determinations was performed with Leu–Val as the analyte and utilizing Leu–Ala as the internal standard. A typical electropherogram is shown in Fig. 7, with 0.7 fmol of Leu–Val migrating before the internal standard at 7.5 min. Results from quantitative studies at two levels are given in Table 6, illustrating quantitative recoveries and good precisions at the levels 10^{-7} *M* (2 fmol injected) and 10^{-6} *M* (20 fmol), 2.4 and 3.8%, respectively; six samples at each concentration level were determined. Two separate standard curves had to be used to get good recoveries; in the broad concentration range 12 standards were used, while 8 standards comprised the curve in the lower concentration range. In each case they were constructed from two independent weighings of the analyte and internal standard. The limit of detection at a signal-to-noise ratio equal

Table 6
Recovery and precision

Range of standard curve (10^7 *M*)	n^c	Added concentration (10^7 *M*)	Determined concentration (10^7 *M*)	R.S.D. (%)	n^d
0.52–29.1[a]	12	1.04	1.20	2.5	6
		10.1	10.1	3.8	6
0.54–2.33[b]	8	1.01	1.02	2.4	6

Analyte: Leu–Val. Internal standard: Leu–Ala ($3.4 \cdot 10^{-7}$ [a] respectively $1.1 \cdot 10^{-7}$ [b] *M*). Linear regression: [a] $y = 0.0061 + 0.122x$ ($r = 0.9997$); [b] $y = 0.0032 + 0.368x$ ($r = 0.9993$).
[c] Number of standards; at six ([a]) and four ([b]) concentration levels from two independent weighings.
[d] Number of samples.

to 3 was determined to be $4 \cdot 10^{-9}$ M (70 amol injected).

4. Conclusions

The main parameters in optimizing the yield for the reaction between dipeptides and NBD-F were determined by chemometric studies applying a fractional factorial design for screening purposes and a central composite face-centred design for response surface modelling. It was found that the reagent excess should be >70 times, the ionic strength should be low (≤ 0.001), optimal pH was 7 and the amount of 2-propanol should be ca. 15%. The derivatization products were anionic, and femto- to attomole amounts of a series of closely related dipeptides could be separated in a background electrolyte of pH 9 with Brij 35 as the micellar agent. The selectivity was improved in the micellar systems compared to applying a neat buffer as the background electrolyte. Furthermore, the use of high pH was necessary to get high fluorescence, and the intensity was further improved about four times in the presence of micelles. Using an argon-ion laser with an output wavelength of 488 nm, mass detection limits of about 70 amol were achieved, and the peptides could be quantified at the level 10^{-7} M (ca. 2 fmol injected) with a relative standard deviation of 2.4%. In future studies the reagent will be applied for assays of larger peptides, like neuropeptides, and possibly also studied for its usefulness in peptide mapping of proteins.

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