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Micropreparative peptide separations by capillary electrophoresis

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Introduction

Peptide analysis is routinely performed using reversed-phase liquid chromatography (RP-HPLC) which achieves separation based on hydrophobicity differences between peptides [1]. Recently, however, capillary electrophoresis (CE) has increasingly been used for peptide analysis. In capillary zone electrophoresis (CZE) the separation mechanism is based mainly on differences in charge to mass ratios and since peptides are amphoteric they are ideally suited to electrophoretic analysis. The different separation mechanism of CZE has provided workers with an excellent complementary tool to HPLC. The provision of such a complementary separation mechanism is extremely useful in characterization of complex biological samples and in particular, where purification of peptides and proteins is often done by preparative HPLC, such an orthogonal technique is essential for purity control [2].

Method development in CZE is primarily focused on optimisation of buffer composition i.e. pH, ionic strength, the physical properties of the buffering ions, and use of additives [3].

One alternative CZ separation mode applicable to peptides is micellar electrokinetic chromatography (MEKC) [4], where peptides are separated by virtue of their hydrophobic interaction with a micellar "pseudo-stationary" phase.

Often, further structural information on the separated peptides has to be obtained. Apart from mass-spectrometry protein sequencing is the method of choice for identification of unknown peptides. Since this cannot be performed on-line after the separation, fraction collection is mandatory. In this paper we show automated fraction collection of peptides in the MEKC mode on a commercial CE instrument. The peptides were identified by N-terminal sequencing in presence of 50 mM SDS.

Materials and Methods

All CE experiments were performed using an HP^{3D}CE system from Hewlett-Packard (Waldbronn, Germany). The system comprises a CE unit with built-in diode array detector and an HP ChemStation for system control, data collection and data analysis.

Protein sequencing was performed on the HP G1005A protein sequencing system using the routine 2.2 chemistry. Collected peptide fractions were loaded onto preped sequencer columns and directly applied to the sequencer.

Results

Using a 75 μm id capillary, 0.5 to 2 pmol of peptide mixtures can be separated. This is more than sufficient for the off-line MALDI-TOF-MS [5], which can easily detect peptides in the femtomole level even in the presence of salt. With the use of a 100 μm id capillary, fraction collection from a single run was able to provide sufficient material for subsequent off-line N-terminal sequencing. This technique was used to collect fractions from a separation of a tryptic digest of the protein GroES (Mr 10,700) [6]. The separation of the peptides was performed in the CZE mode in presence of 105 mM sodium-phosphate buffer at pH of 2.0. With an injection volume of ca. 90 nl, as calculated by the Hagen Poiseuille equation, roughly $\leq$60 pmol of the peptide mixture could be injected. Loading concentrated peptide samples onto a 100 μm "wide-bore" capillary can lead to heat induced precipitation of the sample during analysis. However, capillary thermostatting to 15°C which reduces Joule heating in the capillary, was able of overcome this problem.

Peptide separations using MEKC are usually performed using buffers containing 20 - 100 mM SDS. In Figure 1 the analytical separation of four standard peptides is compared by the CZE and MEKC method with 50 mM SDS. It becomes obvious, that the separation mechanism is completely different using the same capillary, but using different buffers and additives. In figure 2 a preparative separation of the four peptides is shown (50 mM SDS, 20 mM sodium phosphate, pH 9). All peptides were collected by pressure elution into microvials containing 20 μl running buffer. The following steps were performed by the instrument: 1. The voltage was stopped; 2. the outlet vial was changed to the collection vial using the sample tray; 3. on the inlet vial 50 mbar pressure was applied by the built-in air pump for about 40 seconds (depending on peak width and capillary length); 4. the outlet vial was changed back to the outlet buffer vial and 5. high voltage was applied again. The distance from the detector flow cell of the capillary to the capillary outlet is 8.5 cm, which

is 9.7 % of the effective length of the capillary. Therefore the peaks had a delay of 9.7 % of the migration time for migrating to the capillary end. Purity and successful collection of the peptide fractions was confirmed by reinjection of an aliquot from the collected fraction (figure 2, lower traces). The average recovery of the peptides collected by CE was 60-80% as estimated from the reinjected fractions. The collected peptides were identified by sequencing with a yield of 5 - 30 pmol (assuming 50 % initial yield of the sequencer HP G1005A). As an example the sequencing HPLC runs of the collected angiotensin 1 peak are shown in figure 3.

Conclusions

We have shown that peptide separations by CZE on 100 μm capillaries can be readily performed. Potential heat generation, when applying high voltage to the 100 μm capillary, can be reduced by cooling the capillary cassette to 15° C and by starting the separation with a smooth voltage gradient.

Due to the loading and washing processes of samples on the hydrophobic part of the biphasic sequencing column salts, SDS and other buffer solution constituents were removed prior to the Edman degradation.

Use of 100 μm capillaries, or capillaries with even larger inner diameter, for the micropreparative separation of peptide mixtures CE can be a real alternative technique to reversed-phase HPLC, which is presently accepted as the state-of -the-art separation technique for peptide separation. We show here that the MEKC mode can be used as an alternative technique to capillary zone electrophoresis for successful peptide fraction collection.

References

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Legends

Figure 1a:

Capillary zone electrophoresis of four standard peptides (each 1 mg/ml in water: 1. Angiotensin I, 2. Angiotensin II, 3. Xenopsin, 4. Leucine-Enkephalin). Capillary 64 (56) cm x 50 μm with extended light path "bubble cell", temperature 25 °C, run buffer 50 mM sodium phosphate, pH 2.5, 30 KV, injection 100 mbar x s.

Figure 1b:

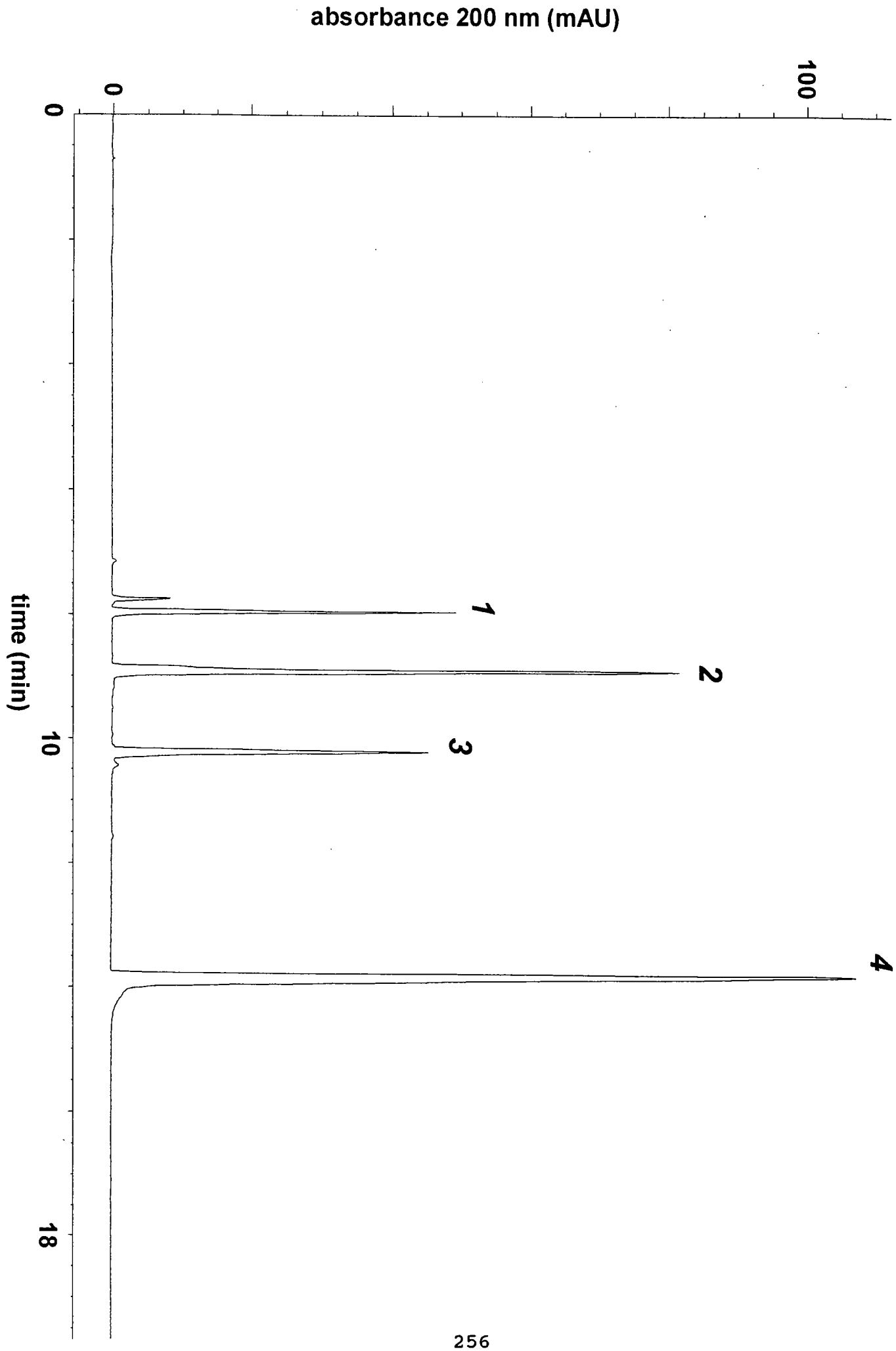
MEKC of the same sample. Capillary 64 (56) cm x 50 μm with extended light path, temperature 20 °C, run buffer 20 mM sodium phosphate, pH 9 with 50 mM SDS, 25 KV, injection 100 mbar x s.

Figure 2:

Upper electropherogram: MEKC preparative run of peptides. Capillary 96 (88) cm x 100 μm, temperature 20 °C, run buffer 20 mM sodium phosphate, pH 9 plus 50 mM SDS, 25 KV, injection 300 mbar x s.
Lower electropherograms: Two reinjected fractions on a 64 (56 cm) x 50 μm capillary. All other conditions the same than above.

Figure 3:

Amino acid sequencing cycles 2,4,7 and 8 of collected Angiotensin I.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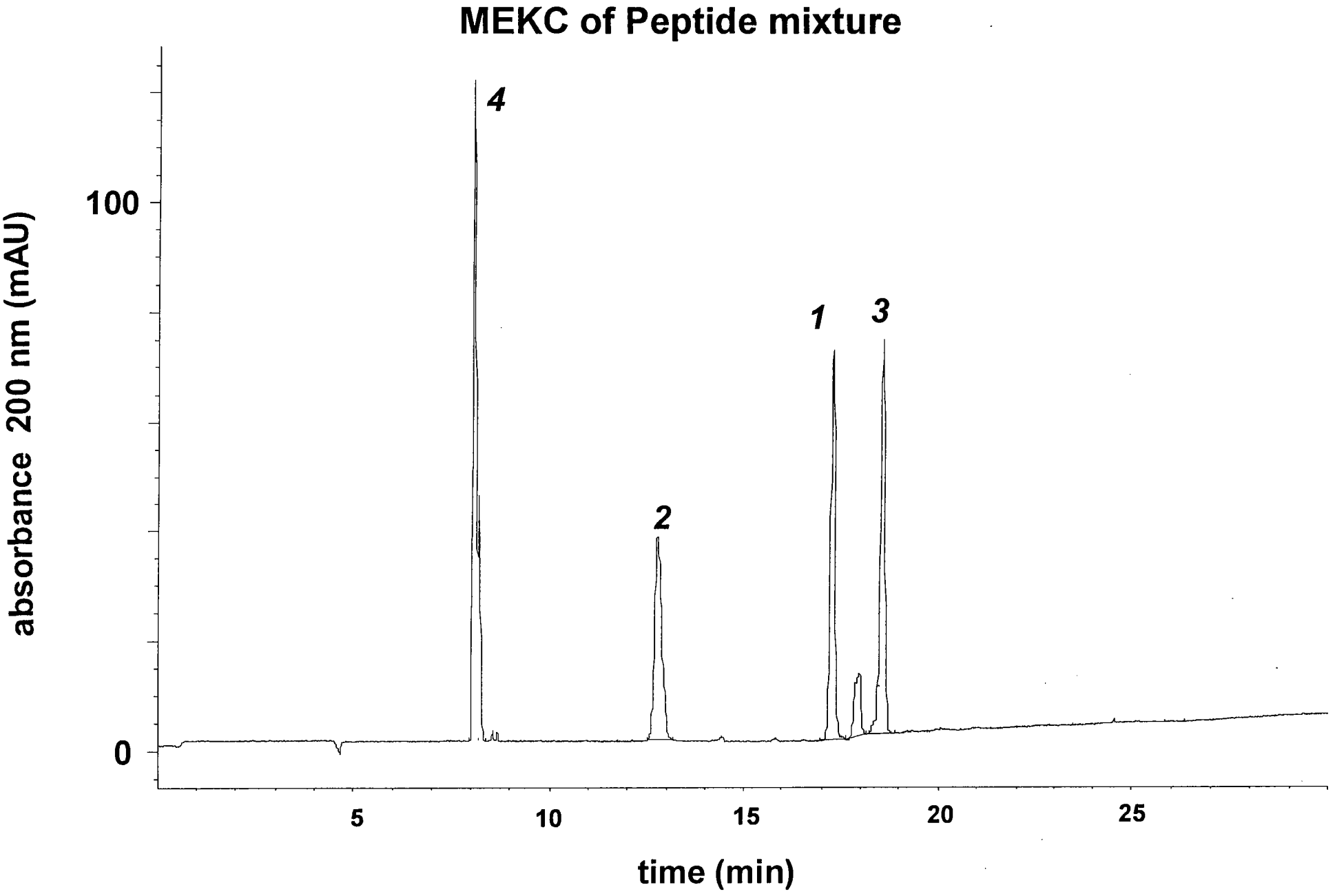
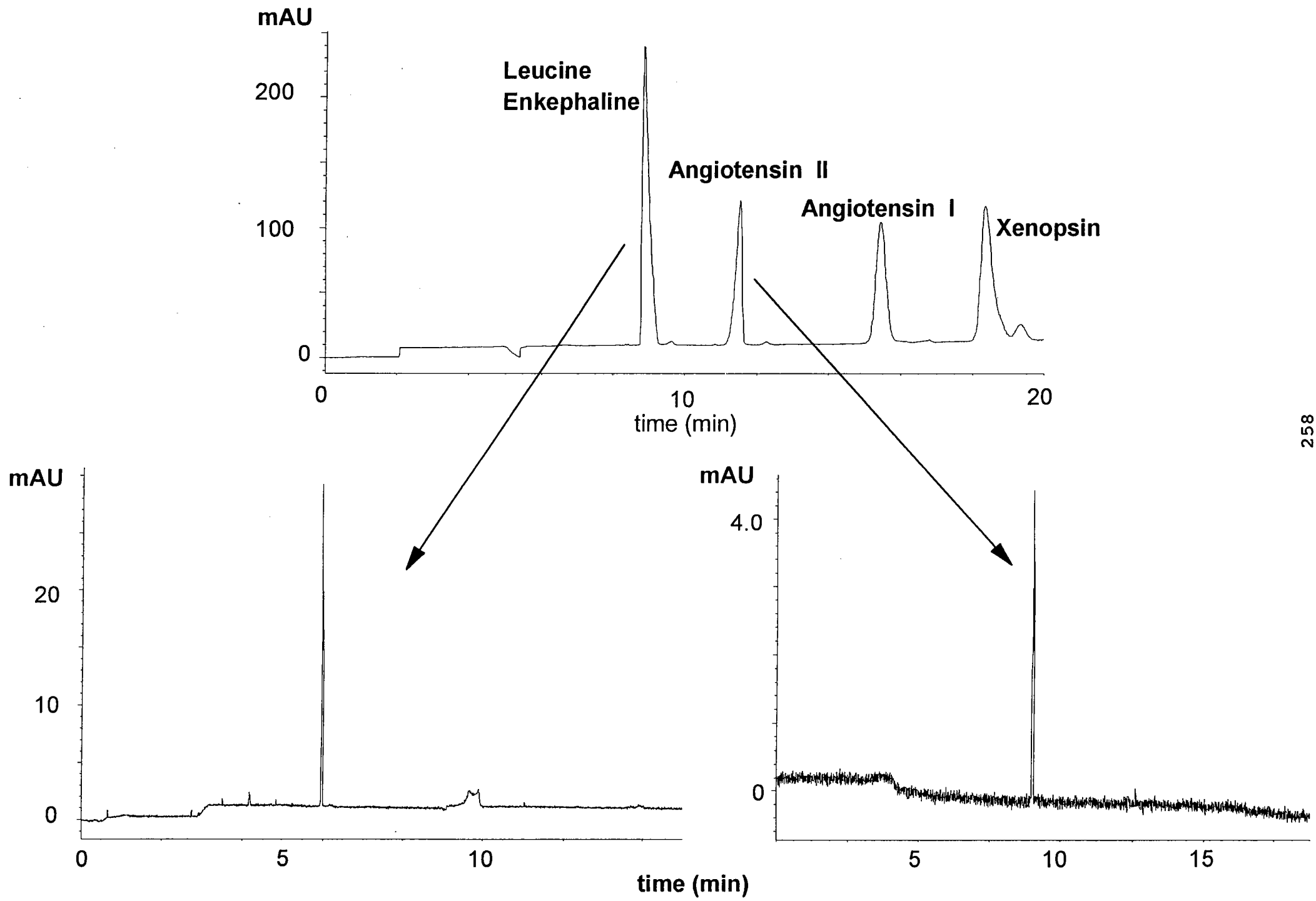
0
100
time (min)
0
10
18
1
2
3
4


MEKC of Peptide mixture
absorbance 200 nm (mAU)
100
0
4
2
1
3
5
10
15
20
25
time (min)


mAU
200
100
0
Leucine
Enkephaline
Angiotensin II
Angiotensin I
Xenopsin
0
10
20
time (min)
mAU
20
10
0
0
5
10
mAU
4.0
0
5
10
15
time (min)

258

Sequence analysis of collected fraction Angiotensin 1