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Journal of Chromatography B, 681 (1996) 99–105

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Separation of common nucleotides, mono-, di- and triphosphates, by capillary electrophoresis

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Abstract

A capillary electrophoretic procedure for the separation of eleven nucleotides, 5′-mono-, di- and triphosphates of adenosine, guanosine, cytidine and uridine, has been developed. All eleven analytes can be separated in a fused-silica capillary (63 cm to the detector, I.D. 75 μm) at 20 kV in a 0.02 mol l^{-1} phosphate–borate buffer (pH 8.0–9.0) with a separation factor $\geq$1. The values of the Offord parameter calculated for individual nucleotides predict that monophosphates will migrate faster than triphosphates, and in turn triphosphates will precede diphosphates. By analogy, faster electroosmotic mobility (lower electromigration) of purine nucleotides (AP, GP) can be explained by a more voluminous structure of purine derivatives (two aromatic rings as compared to pyrimidines). Generally speaking, all compounds separated follow the Offord equation assuming that the triphosphate derivatives are ionized to the third degree forming HL^{3-} anions. This assumption is in agreement with the current knowledge about protolytic equilibria of polyphosphates. The only exception to this rule is faster migration of guanosine-5′-triphosphate (GTP) preceding uridine-5′-monophosphate (UMP) which is ascribed in part to the larger molecule of GTP and the two additional OH-groups bound to the pyrimidine ring of UMP.

Keywords: Nucleotides

1. Introduction

Capillary electrophoresis (CE) underwent a booming development in the past ten years. Due to easy quantitation of separated peaks and the possibility of automation, CE continues to play a more and more important role in analytical biochemistry [1,2]. At present considerable interest is focused on the separation and characterization of nucleosides, nucleotides and short nucleic-acid fragments with the aim to reveal and possibly quantitate delicate changes in the DNA structure [3,4]. Conversely, little attention has been paid so far to nucleotides related to energetic metabolism [30,31].

Previously, at the early stages of deoxy- or ribonucleotide investigation by CE it was demonstrated that the CE separations run strictly according to the base numbers of fragments separated, provided that gel-filled capillaries were used [5].

Conversely, separation of deoxynucleoside-5′-monophosphates as fluorescein–ethylamine conjugates can be achieved in untreated capillaries using laser-induced fluorescence for detection [6]. This approach was selected in order to increase the sensitivity of the assay. The separation of normal and modified nucleoside and nucleoside-3′-monophosphates was reported by Lecoq et al. [7]. There are

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SSDI 0378-4347(95)00535-8

several other papers dealing with the separation of nucleosides, nucleotides and oligonucleosides by micellar electrokinetic capillary chromatography (MECC) and capillary zone electrophoresis (CZE) [8–14], but to our best knowledge none of these reports deal with the separation of nucleoside-5′-mono-, di- and triphosphates, particularly in complex sample matrices.

Successful separation of AMP, cyclic AMP, ADP and ATP at pH 7.0 was described, in a buffer containing 0.5% ethylene glycol [15]. Other authors [16,17] used silylated capillaries for the separation of selected oligonucleotides and/or nucleosides (both 5′- and 3′-) triphosphates. These methods though applicable in practice are a bit complicated as successful separation of the strongly anionic triphosphates requires suppression of the endo-osmotic flow (EOF), e.g. by silylating the internal surface of the capillary by means of γ-methacryloxypropyltrimethoxysilane followed by polyacrylamide coating. This approach represents a considerable complication of the method: silylated capillaries are not commercially available and because of the coating procedure there is no guarantee of an easy transfer of the results obtained from one capillary to another. Moreover, both methods do not involve a complex mixture of mono-, di- and tri-derivatives and as a consequence of abolishing the EOF the separation must be run at high voltage in reversed polarity mode. On the other hand, a considerable advantage of methods using silylated capillaries [16,17] is the low absolute detectable amount of nucleoside triphosphates in CE as compared to HPLC. The minimum detectable concentration, however, is only comparable with HPLC, being in the order of 0.001 mg/ml (ca. 10^{-6} *M*) [16]. MECC with both anionic (SDS) and cationic (DTAB) surfactants was used to partially resolve CTP, UTP, GTP and ATP [18].

To our best knowledge there are only a few papers dealing with a simple separation of nucleoside-5′-phosphates either for resolving ^{32}P-labelled ATP and GTP [19] or dealing with the separation of phosphonate analogues of uridine [20] or adenosine [21].

Several other methods besides CE exist for the measurement of nucleoside triphosphates and their metabolites including enzymatic assays [22], gas chromatography [23] and HPLC [24]. Of the mentioned approaches only HPLC has been reported to measure the common nucleoside and deoxynucleoside triphosphates [24], but the method requires about 60 min to elute all the components of the nucleoside triphosphate mixture.

The aim of the present communication was to develop a simple and rugged method for the separation of nucleoside mono-, di- and triphosphates, which would be short enough to allow its application in bio-process monitoring.

2. Experimental

2.1. Instrumentation

The experiments were performed with SpectraPhoresis 504 apparatus from Thermo Separation Products (TSP, Riviera Beach, FL, USA), controlled with PC 1000 Version 2.6 software (supported on OS2 2.1), equipped with the in-line, variable-wavelength detector set to 254 nm (though detection at 220 nm offered also satisfactory results).

The separations were run at 20 kV (30–40 μA) with an untreated fused-silica capillary, I.D. 75 μm, fitted into the TSP cartridge. Total capillary length was 70 cm (63 cm to the detector).

An uncoated silica capillary was conditioned by sequentially using it for 5 min in water, 0.1 *M* NaOH, water and finally the buffer to be used for the subsequent separation. This procedure was also used to recondition the capillary after every 5 runs.

2.2. Reagents

Potassium dihydrogen phosphate, sodium tetraborate and potassium dihydrogen citrate were obtained from Merck (Darmstadt, Germany) and were of p.a. purity. All background buffers were filtered through a 0.45-μm filter.

Adenosine-, guanosine-, uridine-5′-mono-, di-, triphosphates, cytidine-mono and diphosphates (sodium salts) were purchased from Sigma (St. Louis, MO, USA) and were of 97% purity grade. Samples were prepared by dissolving 6–16 mg of each nucleotide in 5 ml of MilliQ water and stored below 4°C.

The standard 11-membered mixture for CE separation was prepared by delivering 10 μl of each

standard into the vial which contains 1 ml of water. All samples were injected hydrodynamically for 1 s (injected volume approx. 4 nl, applied vacuum 10 kPa).

3. Results

Since preliminary experiments with a set of Kolthoff-Vleeschhower buffers indicated that the EOF in our capillary increased up to pH 7.0 and practically did not change with an additional increase up to pH 9.8 (Fig. 1) we have followed the separation of the investigated set of nucleotides in neutral (pH 7.0) or weakly basic phosphate–borate buffers (0.02 mol l^{-1}, pH 8.0–9.0) with the idea that under such conditions the runs may be fast enough to be applicable to process control. Routinely the separations were done at pH 8.5.

As demonstrated in Fig. 2, all components of the 11-membered mixture were separated at pH 8.0 and 9.0 (with a separation factor ≥1 for any consequent pair of peaks). At pH 7.0 the separation was still acceptable, though the separation of the third through the sixth peaks, i.e. cytidine monophosphate (CMP) with guanidine triphosphate (GTP) and uridine monophosphate (UMP) with adenosine triphosphate (ATP), was incomplete (Fig. 2c).

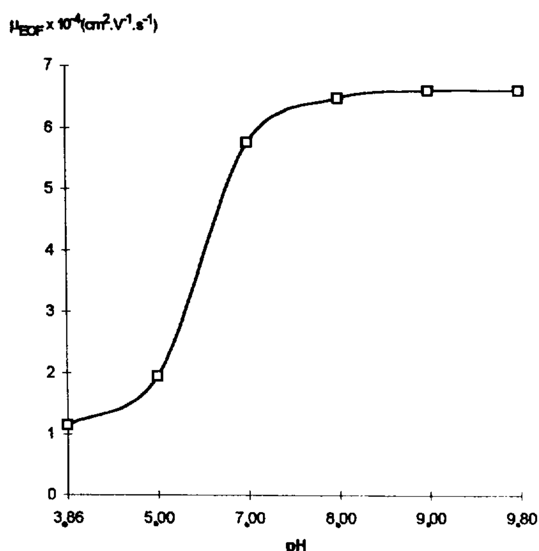


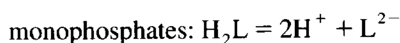
Fig. 1. Effect of pH on electroosmotic flow. Conditions: benzylalcohol as neutral marker; fused-silica capillary, 70 cm × 75 μm I.D.; 20 kV; 25°C; detection at 254 nm; hydrodynamic injection (1 s). Buffers (0.02 mol l^{-1}): citrate–borate (pH 3.8–5.0), phosphate–borate (pH 7.0–9.0), phosphate–NaOH (pH 9.8).

As also shown in Fig. 2 the anodic mobility increases from guanosine to adenosine, cytosine and uridine derivatives, the uridine derivatives being the fastest and guanosine derivatives the slowest in their motion towards the anode; consequently guanosine phosphates appear first before the detection window while the uridine derivatives emerge last. With all phosphate derivatives, monophosphates always exhibited the lowest anodic mobility followed by their respective triphosphates. Diphosphates migrated with the highest anodic speed. In the interval of pH 8.0–9.0 (Fig. 2a,b) a complete separation was achieved within less than 15 min. At pH 10.0 (Fig. 2d) the separation time was slightly shorter, though there was insufficient separation of uridine triphosphate–guanidine diphosphate (UT–GD) and cytidine diphosphate–uridine diphosphate (CD–UD).

As expected, the migration order of the individual nucleotides is not just a mirror image to that obtained by separation at pH≤4 in capillaries coated with linear polymers [16,17,25] or in PTFE capillaries [26]. It was found that in the absence of EOF [17], triphosphates migrate faster than diphosphates which in turn migrate faster than monophosphates. Furthermore, in all three structural groups, the migration order of a given derivative was: uridine-, guanosine-, adenosine- and cytidine-phosphates. Some exceptions and irregularities, however, occurred and were explained as the consequence of small differences in pK_a values [17] or in molecular masses [25] of individual nucleotides.

With regard to the separation of nucleotides in fused-silica capillaries at pH 8.0–9.0, it should be emphasized that the migration order observed cannot be simply related to protolytic equilibria of esterically-bonded phosphoric acid defined by protolytic constants, pK_a, and the pH value of the run buffer.

Assuming complete dissociation of all ionizable protons of bonded phosphoric acid, which can be expressed by the following equilibria:



monophosphates: $H_2L = 2H^+ + L^{2-}$

diphosphates: $H_3L = 3H^+ + L^{3-}$

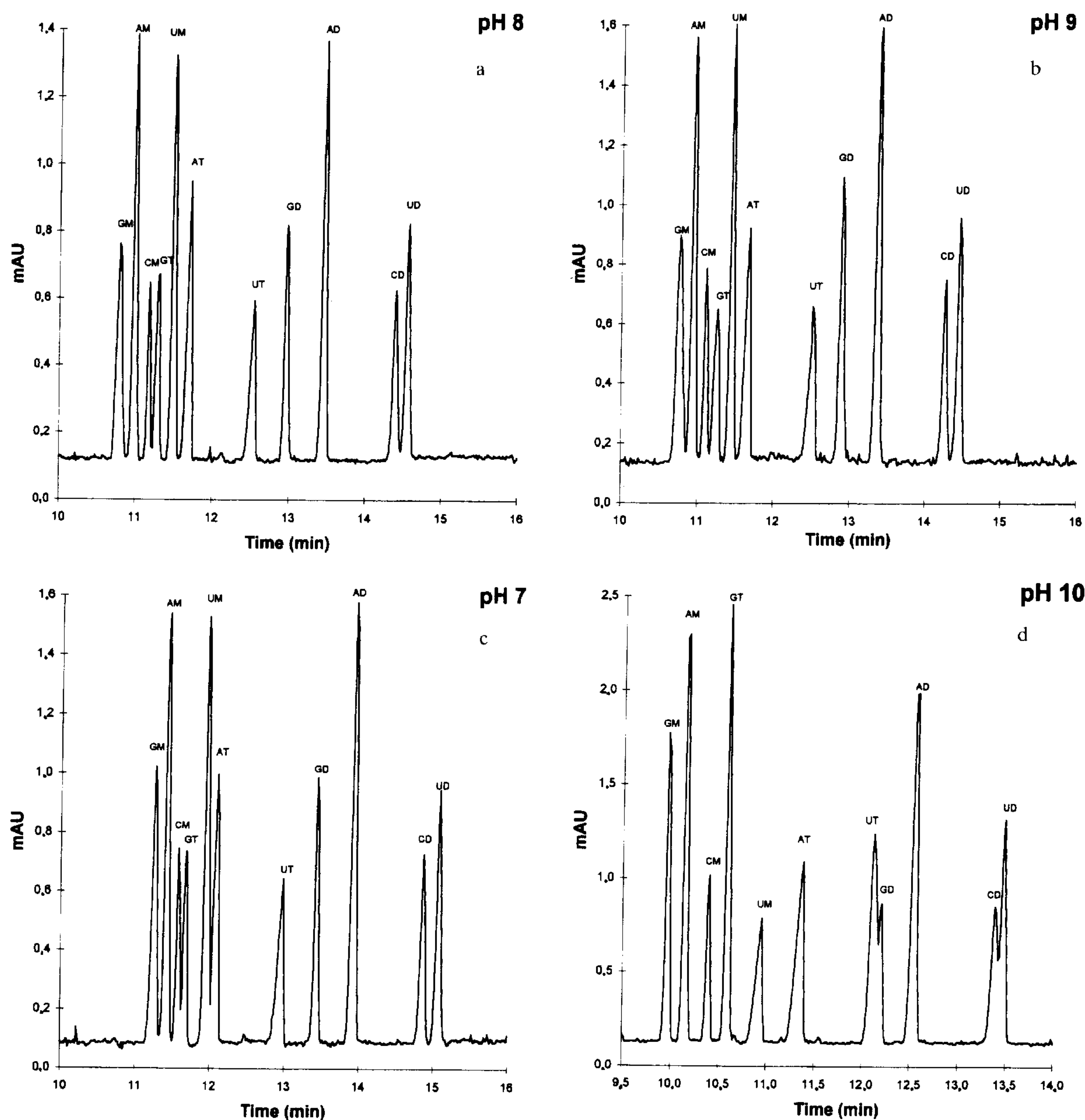


Fig. 2. Electropherograms of 11-membered mixtures of nucleoside-5′-phosphates. Conditions: individual sample concentration, 10 μg ml^{-1}, phosphate-borate buffer, 0.02 mol l^{-1} (adjusted to pH 10.0 with NaOH). Other conditions are the same as described in the legend to Fig. 1.

triphosphates: $H_4L = 4H^+ + L^{4-}$

(where L represents deprotonated anionic species of related nucleotides) the migration sequence should be mono-, di- and triphosphate, the latter exhibiting the fastest anodic movement. However, in the experiments we consistently observed an altered migration sequence: while monophosphates were observed first in the detector's window, tri- and diphosphates were interchanged, the latter exhibiting the most rapid anodic migration. In our view it is reasonable to assume that the dissociation of nucleoside triphos-

phates is incomplete and results in HL^{3-} not in L^{4-}. This is in agreement with the present knowledge about protolytic equilibria of polyphosphates where the terminal OH group is a weaker acid when compared to the other OH moieties present in the molecule. Furthermore, the impact of hydrogen

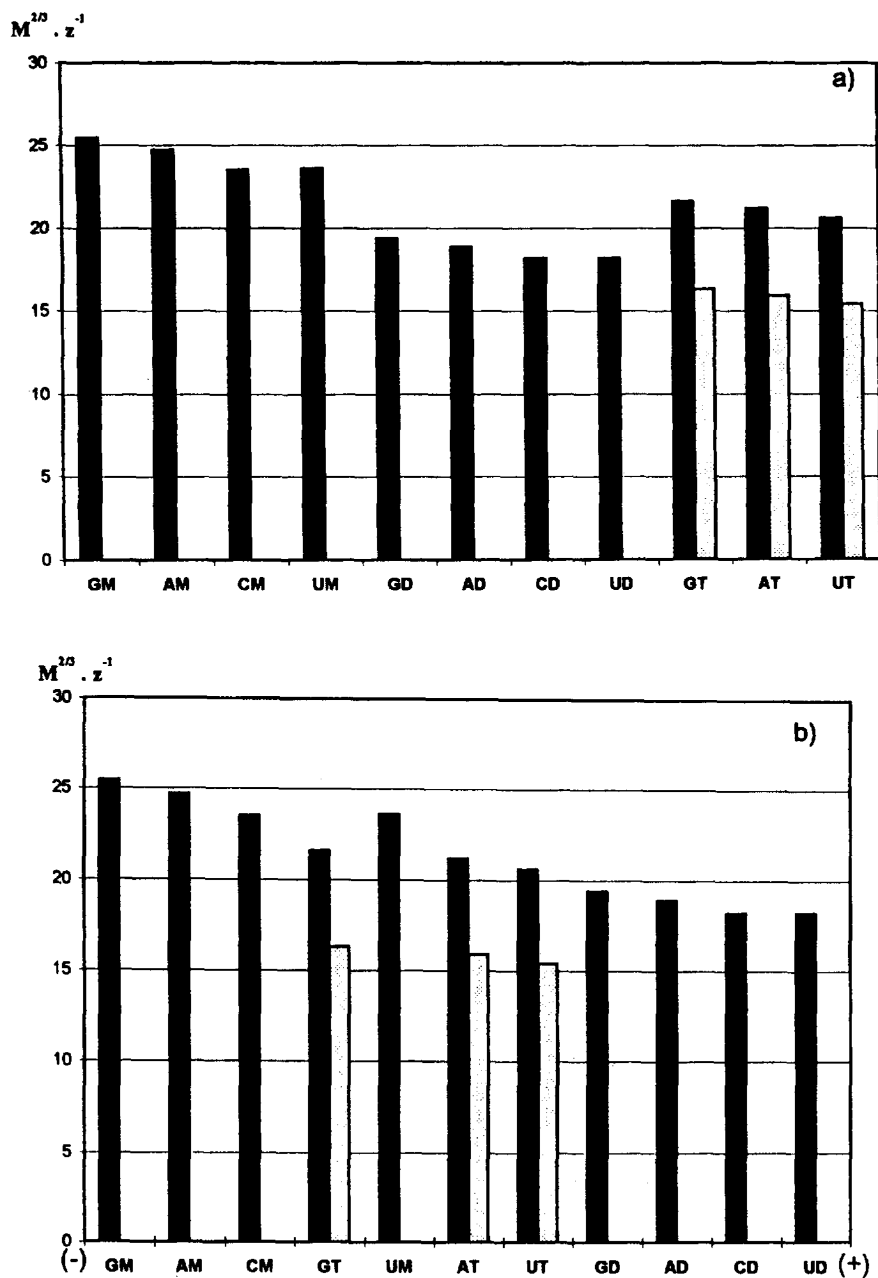


Fig. 3. Offord parameters of nucleoside-5′-phosphates. Mass-charge ratio calculated from Offord's equation [27]: $M^{2/3}/z$; (a) nucleotide order according to the increasing mass-charge ratio assuming complete dissociation, i.e. monophosphates as L^{2-}, diphosphates as L^{3-} (black columns) and triphosphates as/or L^{4-} (dotted columns) HL^{3-} (black columns); (b) nucleotide order according to their migration sequences improving the dissociation of triphosphates to HL^{3-} (black columns).

Table 1
Repeatability of migration time (MT) of individual nucleotides in the mixture

Compound	Mean value of MT (n=10, min)	S.D	R.S.D. (%)
GMP	10.809	0.080	0.74
AMP	10.988	0.078	0.71
CMP	11.130	0.077	0.69
GMP	11.277	0.084	0.74
UMP	11.518	0.087	0.76
ATP	11.654	0.079	0.68
UTP	12.479	0.085	0.68
GDP	12.880	0.105	0.82
ADP	13.346	0.101	0.76
CDP	14.205	0.092	0.65
UDP	14.412	0.120	0.83

Conditions: 0.02 mol l^{-1} phosphate–borate buffer (pH 8.5); fused-silica capillary, 70 cm × 75 μm I.D., reconditioned after 5 runs, hydrodynamic injection (1 s). Separation conditions: 20 kV, 25°C.

bonds on the distribution of individual ionic forms must not be ignored.

A similar conclusion can be reached by comparing the migration order with the Offord parameter [27] expressed as ratio of the molecular mass (M) of the solute and the effective charge (z) of the particle in the form: $M^{2/3}/z$. Were it so that triphosphates yielded the L^{4-} anion, their Offord parameters would be changed (decreased) to the value shown by dotted columns in Fig. 3a. This would move them to the right hand side of the Fig. 3a and, consequently, make them last to appear before the detector. While in Fig. 3a the nucleotides were ordered according to their decreasing Offord parameter, in Fig. 3b they are ordered according to the observed sequence in CE. It is evident at the first sight that they indeed follow the Offord rule, if following anionic species of studied nucleotides are supposed: monophosphates as L^{2-}, triphosphates as HL^{3-} and diphosphates as L^{3-}. There is, however, a single exception: uridine monophosphate (UMP) migrates slower than guanosine triphosphate (GTP). This may be explained by structural differences between pyrimidine and purine derivatives, the latter being bigger by an aromatic ring.

Repeatability of the migration time for each nucleotide fluctuated in the range of 0.6–0.8% as shown in Table 1. The satisfactory migration time repeatability values presented here can (under the conditions of the fast EOF) be obtained only if proper equilibration of the capillary is done at least once in 5–6 runs. Also, it is mandatory that the contents of the anodic and cathodic jars be replaced with a fresh buffer.

Because the separation procedure reported here is to be exploited for the quantitation of these analytes in the *Candida utilis* cells (data not shown), appropriate calibrations were done for all 11 derivatives investigated. The dependence of the peak area on the

Table 2
Peak height–concentration relationship

Compound	Equation of regression line: μg vs. μAU		Detection limit (μg)	Concentration in stock standard solution (μg/ml)
	Slope	y-Intercept		
AMP	87.7	−14.4	0.62	21.0
ADP	97.2	−191	3.8	22.2
ATP	49.6	−9.4	1.2	22.2
GMP	69.2	11.0	0.88	16.6
GDP	87.6	−72.2	1.7	13.0
GTP	52.0	−29.5	2.2	15.6
CMP	38.8	30.7	2.2	17.6
CDP	44.1	−80.2	2.7	22.2
UMP	56.1	−54.4	2.3	32.2
UDP	77.4	−97.9	3.0	17.6
UTP	22.0	−5.8	1.8	28.0

Conditions: 0.02 mol l^{-1} phosphate–borate buffer (pH 8.5); uncoated fused-silica capillary, 70 cm × 75 μm I.D. (63 cm to the detector), 20 kV, 30 μA, hydrodynamic injection (1 s), detection at 254 nm. Calibration solutions were prepared by diluting 8, 6, 4, 2 and 1 ml of the stock standard mixture in 10 ml volumetric flasks. Calibration mixtures were prepared and run in triplicate.

concentration of a particular derivative was linear between 3 and 30 $\mu g/ml$. The range and parameters of the calibration lines are summarized in Table 2, along with the detection limit values (taken as 3-times noise level).

4. Conclusion

From the data presented it follows that the separation of nucleotides at pH 8.0–9.0 in an untreated fused-silica capillary can serve as an effective alternative for a sensitive phosphorylated nucleoside assay, provided that the EOF is carefully controlled. This approach can easily replace the currently available enzymatic [28] or HPLC [29,30] methods. In this context, it is worthy to mention some advantages inherent to capillary electrophoresis, such as small demands on sample size, simplicity of operation, low solvent consumption and short analysis time.

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