to an electrophoretic separation chip is one possible answer. However, there is no reason why reactions cannot be carried out on such devices. Since it does not require the investment of a large chemical plant, the reactions can be performed where required, thus reducing the need to transport hazardous chemicals across countries. Since many reactors can be constructed on a single chip, and many chips located in the same area, it is evident that this technology will provide hazardous or chemically unstable chemicals where they are required.

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Nonaqueous Capillary Electrophoresis

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Electrophoresis is a separation technique that is normally performed in an aqueous environment. This is due to the fact that the separation mechanism is based on the difference in migration rate of charged species in an electric field. Species (ions/molecules or particles) with a difference in their charge over size ratio will exhibit a difference in migration rate. Most charged species are fairly soluble in aqueous media and thus water is the most obvious solvent for electrophoresis. However, in a number of nonaqueous solvent systems, it is possible to obtain sufficient conductivity to perform electrophoresis. If such systems are utilized with the technique of capillary electrophoresis, a number of advantages compared to aqueous systems are obtained in the separation of small molecules. Nonaqueous electrophoresis of biopolymers like polysaccharides, nucleic acids and proteins is not of practical use due to lack of solubility of such molecules in organic solvents.

Nonaqueous Capillary Electrophoresis

Only a few attempts to perform nonaqueous paper electrophoresis have been described and these articles were reviewed in 1978. In 1984 nonaqueous capillary electrophoresis (NACE) was briefly mentioned in a single publication, but not utilized further. However, since 1993 the use of nonaqueous media for capillary electrophoresis has seen renewed interest in the separation of drug substances due to the high separation selectivity obtained in these systems.

The electrophoretic migration of the solutes is influenced by the nature of the solvent or solvent mixture used for the electrophoresis medium in three main ways:

1. The mobility may change due to changes in the size of the solvated ion.
2. The dielectric constant of the organic solvent may influence the equilibrium of the protolytic dissociation. The higher the value of the dielectric constant, the higher the degree of ionization of acids and bases.
3. The acid–base property of the solute, expressed by its pK_a value, may change due to the differentiating effect of many organic solvents.

The latter effect of the three is the most significant, as the dissociation constant, K_a, may change many orders of magnitude for different solvents.

The increased selectivity of separation in organic solvents compared to aqueous systems is due to the fact that the levelling effect of water is eliminated. If

Table 1 Classification of organic solvents according to their Brønsted acid–base behaviour

Solvent designation		*Relative acidity*	*Relative basicity*	*Examples*
	Neutral	+	+	*MeOH*, glycerol, phenol, *tert*, butyl alcohol
Amphiprotic	Protogenic	+	−	Sulfonic acid, formic acid, *acetic acid*
	Protophilic	−	+	Liquid ammonia, *FA*, *NMF*
	Dipolar protophilic	−	+	*DMSO*, *DMF*, tetrahydrofurane, 1,4-dioxan, pyridine
Aprotic	Dipolar protophobic	−	−	*MeCN*, acetone, nitrobenzene, sulfolane, *PC*
	Inert	−	−	Aliphatic hydrocarbons, benzene, 1,2-dichlorethane, tetrachloromethane

− indicates weaker and + indicates stronger acid or base than water. DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide: FA, formamide; MeCN, acetonitrile; MeOH, methanol; NMF, *N*-methylformamide; PC, propylene carbonate. Solvents *in italic* are the ones that are preferred for NACE. Reproduced with permission from Tjørnelund J and Hansen SH (1999) *Journal of Biochemistry and Biophysical Methods* 38: 139–153.

strong acids or bases are dissolved in water, they all show up with about the same acid or base strength. If the same acids or bases are dissolved in organic solvents they will exhibit very different protolytic behaviour depending on the degree of dissociation, which again depends on the solvent in question.

Important factors influencing the choice of organic solvent or solvent mixture for a given separation are volatility, the dissolving power towards suitable electrolytes, viscosity and dielectric constant, UV transparency and, last but not least, the effect on the separation selectivity of the system. Information on the viscosity and volatility, the auto protolysis constant, the dielectric constant at standard conditions and the UV transparency of the neat solvents may be found in the literature. In contrast, data on solvent mixtures and systematic studies of how to choose solvents and electrolytes in order to control the selectivity of the electrophoretic system are limited and thus the choice of separation media is still a matter of trial and error. Solvents may be classified according to their Brønsted acid–base behaviour; a simplified version of this classification is shown in **Table 1**.

Practical Considerations

Choice of Organic Solvent

The physical chemical properties of the organic solvents preferred for NACE are given in **Table 2** and, as mentioned above, the physical constants have a major impact on the choice of solvent or solvent mixture for a given electrophoretic separation. Some of the more practical considerations are the chemical resistance of parts in the CE instrument towards the solvent, the volatility of the solvent, the solvating power of the solvent towards electrolytes, the UV transparency and the viscosity of the solvent.

Table 2 Physicochemical parameters of selected solvents

Solvent	*Viscosity, η (cP)*	*Dielectric constant, ε*	ε/η	*pK_{auto}*	*T_{boil} (°C)*	*UV cutoff (nm) (1 cm cuvette)*
Water	0.89	78.4	89.9	14	100	<200
FA	3.3	111	33.6	16.8	210	275
NMF	1.65	182	110.3	10.7	182	275
DMF	0.8	36.7	45.9	29.4	153	260
DMSO	1.99	46.7	23.4	33.3	189	260
MeOH	0.544	32.7	60.6	17.2	65	205
PC	2.5	64.4	25.7	Not detected Protolysis	242	200–230
MeCN	0.34	37.5	110.3	Not detected Protolysis	82	200–230
Glycerol	945	42.5	0.045	—	290	205
Acetic acid	1.04_{30}	6.152	5.91	14.45	118	—

All values are at 25°C unless otherwise stated in subscript. For abbreviations, see Table 1. Reproduced with permission from Tjørnelund J and Hansen SH (1999) *Journal of Biochemistry and Biophysical Methods* 38: 139–153.

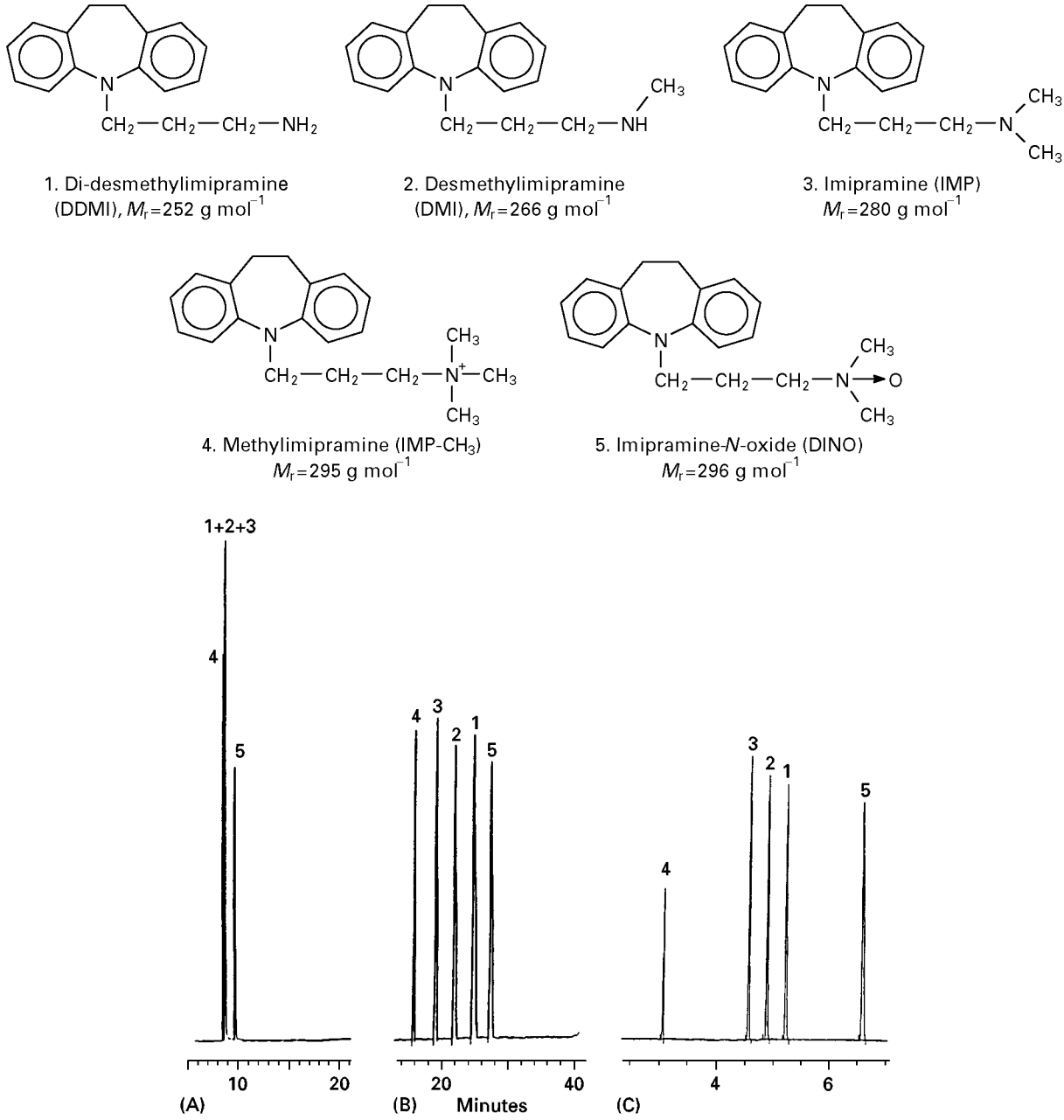

Figure 1 Electropherograms of imipramine and four derivatives. (A) 50 mmol L^{-1} 6-aminocaproic acid pH 4.0; (B) 50 mmol L^{-1} 6-amino caproic acid pH 4.0 with 25 mmol L^{-1} of 3-(*N*,*N*-dimethylmyristylammonium) propanesulfonate and 15 mmol L^{-1} of Tween® 20 added. Apparatus: Quanta 4000. Conditions: 64 cm (56 cm to the detector) × 75 μm i.d. capillary, hydrostatic (10 cm) injection for 15 s, ambient (27–30°C), 20 kV (62 μA) and UV detection at 214 nm. (C) 25 mmol L^{-1} ammonium acetate and 1 mol L^{-1} acetic acid in acetonitrile. Apparatus: HP3DCE instrument. Conditions: 64 cm (55.5 cm to the detector) × 50 μm i.d. capillary, injection of 3 s at 5 kPa (50 mbar), 25°C, 25 kV (7 μA) and UV detection at 214 nm. Adapted with permission from Bjørnsdottir I, Tjørnelund J and Hansen SH (1996) Selectivity enhancement in capillary electrophoresis using nonaqueous media. *Journal of Capillary Electrophoresis* 3: 83–87.

Solvents with a high vapour pressure and thus a high volatility (e.g. methanol (MeOH) and acetonitrile (MeCN)) may be inconvenient for automated analysis in some instruments due to problems with evaporation of the electrophoresis medium from the run buffer vials as well from the sample vials. In CE the detection is often performed by measuring the UV absorbance of the analyte at a relatively short wavelength (e.g. at 214 nm or below) in order to increase the sensitivity. However, many organic sol-

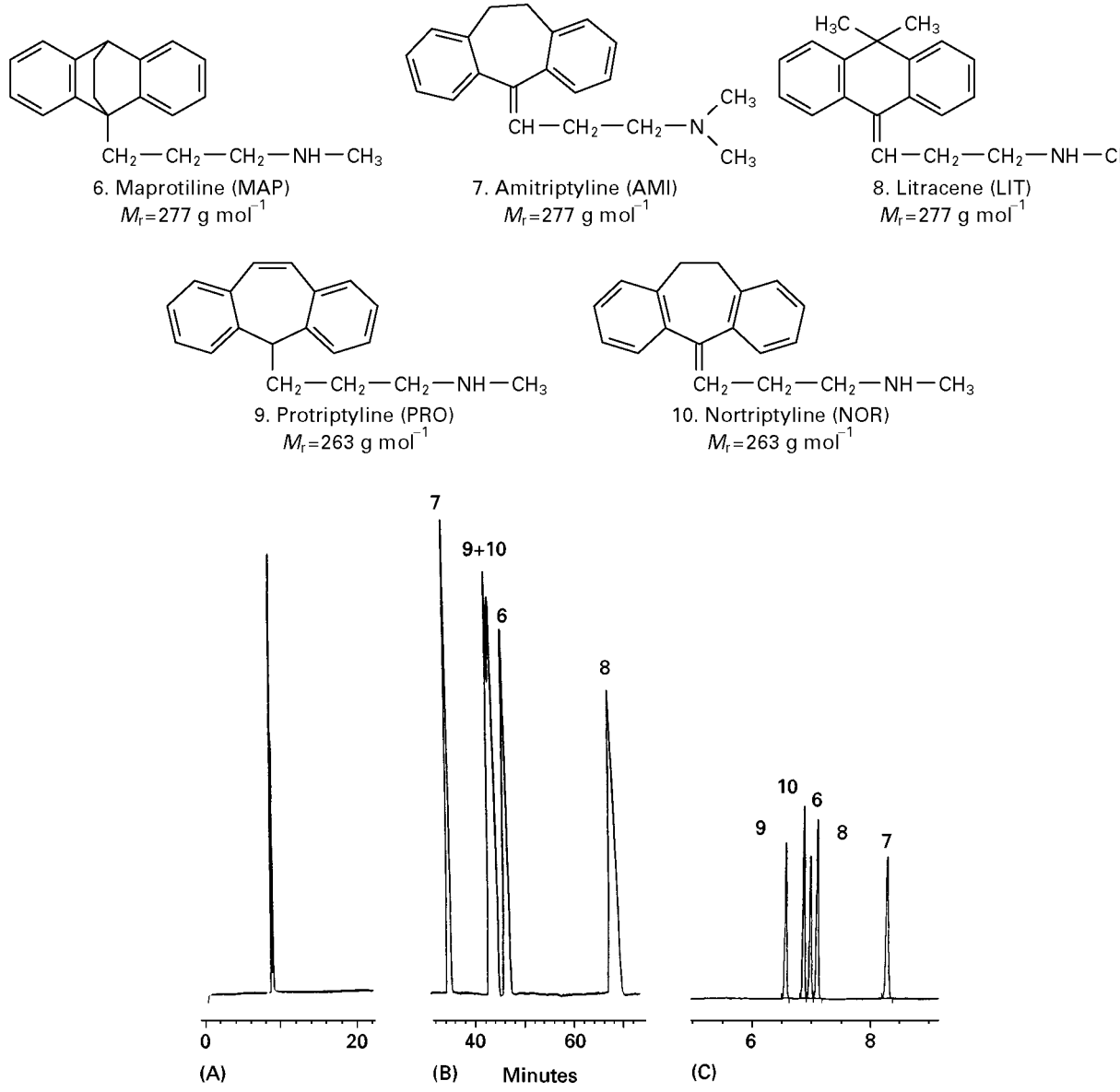

Figure 2 Electropherograms of five basic drugs with equal or very similar mass over charge ratio. (A) 50 mmol L^{-1} 6-aminocaproic acid pH 4.0; (B) 50 mmol L^{-1} 6-amino caproic acid pH 4.0 with 25 mmol L^{-1} of Tween® 20 added. Apparatus and conditions as in Figure 1A. (C) 25 mmol L^{-1} ammonium acetate and 100 mmol L^{-1} sodium acetate in methanol + acetonitrile (1 : 1 v/v) and 25 kV (23 μA). Apparatus and other conditions as in Figure 1C. Adapted with permission from Bjørnsdottir I, Tjørnelund J and Hansen SH (1996) Selectivity enhancement in capillary electrophoresis using nonaqueous media. *Journal of Capillary Electrophoresis* 3: 83–87.

vents have a UV cutoff at 214 nm or above (Table 2). Nevertheless, solvents like MeCN and MeOH may be used for measurements performed at a wavelength as low as 200 nm as the light path through the capillary is very short compared to the 1 cm cuvette used for the determination of the UV cutoff wavelength. The amides and dimethylsulfoxide can only be used when detection at wavelengths above *c.* 245 nm is sufficient of the application.

On the positive side, organic solvents often intensify the fluorescence relative to what is observed for given solutes in aqueous media. This has been used to decrease detection limits in NACE for analysis of tetracyclines in biological matrices.

Choice of Electrolyte

The choice of electrolyte is important and will influence the separation. However, due to the low solubility of many electrolytes in organic solvents, it can be difficult to find a suitable electrolyte. The more polar solvents, like MeOH, DMSO, formamide, *N*-methylformamide and *N*,*N*-dimethylformamide, possess

a good solvating power towards the electrolytes commonly used in NACE. So far, ammonium acetate has been the most frequently used electrolyte in NACE systems and acetic acid or sodium acetate have often been used in combination with ammonium acetate in order to adjust the acid–base properties of the electrophoresis medium. Quaternary ammonium salts have also been used a number of times with success, e.g. in the separation of phenols and carboxylic acids. More rarely, Tris, magnesium acetate, citric acid, formic acid, trifluoroacetic acid and methanesulfonic acid have been used.

When coupling CE to mass spectrometry (MS), it is an advantage to choose a volatile electrolyte, e.g. ammonium acetate, in order to limit background noise or cluster ion formation.

Other Additives

A number of polyalcohols and surfactants such as the Tweens® have been used as additives. Their primary function is to decrease the electroosmotic flow (EOF) and thus prolong the time for electrophoretic separation.

Also chiral separations are possible in NACE using either cyclodextrines or chiral counter ions as additives.

Reversal of EOF

The separation of anionic solutes in CE may lead to extended time of analysis due to their migration in the direction opposite to EOF. One method of decreasing the analysis time is to reverse the EOF, thus making the anions migrate in the same direction as the EOF. In aqueous CE, the addition of long alkyl chain trimethylammonium ions is used for this purpose, e.g. in the analysis of inorganic anions and phenols. This principle may also be used in NACE. However, the long alkyl chain trimethylammonium ions are not able to form hemimicelles at the inner capillary surface when using nonaqueous solvents and thus the EOF is not reversed. Addition of the polycation hexadimethrine bromide to the nonaqueous electrophoresis medium may result in suitable and stable systems with reversed EOF, even when used at fairly low concentrations (0.001–0.05%).

Applicability of NACE

In CE the separation of solutes is due to differences in the charge over size ratios and thus very similar substances may be difficult to separate in aqueous CE unless special mechanisms like micellar electrokinetic chromatography (MEKC) are involved. Of course this involves addition of one or more surfactants.

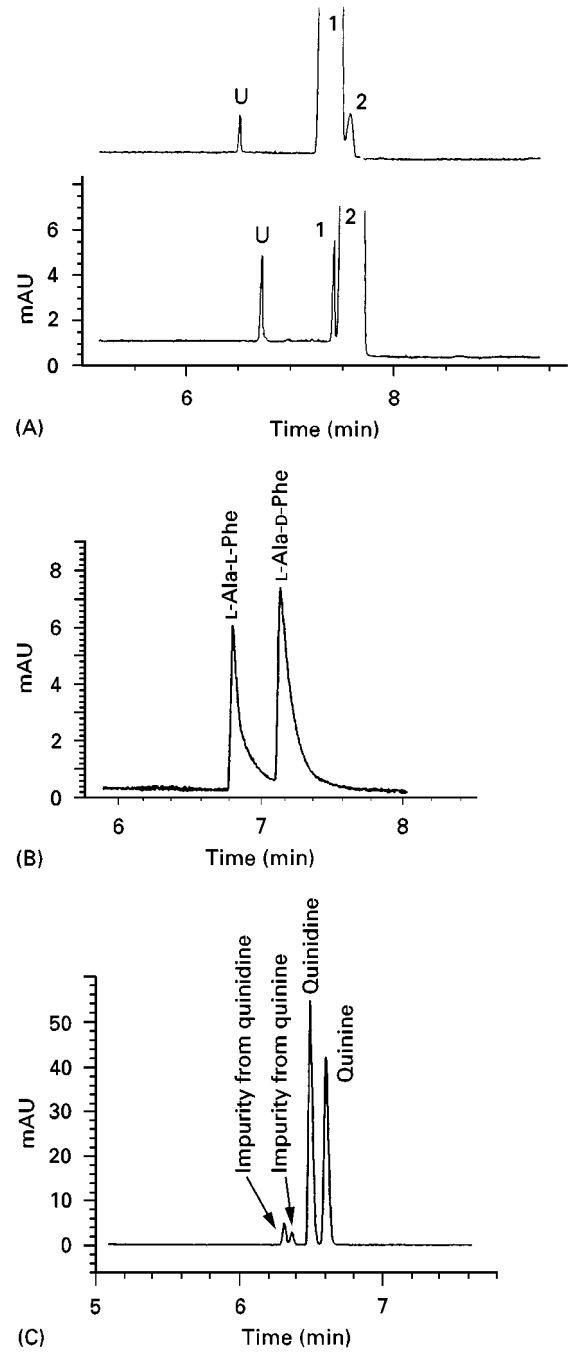

Figure 3 Electropherograms of *cis-trans-* and diastereo-isomers. (A) Separation of *cis-* and *trans*-flupenthixol decanoate using 50 mmol L^{-1} ammonium acetate and 1 mol L^{-1} acetic acid in methanol + acetonitrile (1 : 1, v/v), above: *cis*-flupenthixol decanoate with 0.5% *trans*-isomer added; below: *trans*-flupenthixol decanoate. Conditions: 64 cm (55.5 cm to the detector) × 50 μm i.d. capillary, injection for 3 s at 5 kPa (50 mbar), 25°C, 30 kV (9 μA) and UV detection at 230 nm. Test solution: 5.0 mg mL^{-1} of the sample in methanol + acetonitrile (1 : 1 v/v). Peak identity: 1, *cis*-flupenthixol decanoate; 2, *trans*-flupenthixol decanoate; U, unknown. (B) Separation of dipeptides (diastereomers); (C) separation of quinine and quinidine (diastereomers). Conditions as in (A) with a detection wavelength of 214 nm. Adapted with permission from Hansen SH, Bjørnsdottir I and Tjørnelund J (1997) Separation of cationic *cis-trans* (Z-E) isomers and diastereomers using nonaqueous capillary electrophoresis. *Journal of Chromatography A* 792: 49–55.

Table 3 Applications of NACE in analysis of food, pharmaceuticals and biological fluids

Solvents	*Electrolytes*	*Analytes*
Applications within food		
NMF-dioxane (1 : 1 v/v)	40 mmol L^{-1} Tris, 2.5 mmol L^{-1} anthraquinone-2-carboxylic acid	Free saturated long chain fatty acids (*n*-C_{14}-*n*-C_{26}). Separation of dimeric and trimeric acids and hydrogenated fish oil
NMF	500 mmol L^{-1} magnesium acetate tetrahydrate	Tetracycline (TC), oxytetracycline (OTC), chlorotetracycline (CTC), demeclocycline, 4-epitetracycline, anhydrotetracycline, 4-epianhydrotetracycline, and desmethyltetracycline TC, OTC and CTC in milk and plasma
Propylene carbonate	Tetraalkylammonium ions, long chain trimethylammonium ions 20 mmol L^{-1} tetradecylammonium bromide (vitamin K_1 and preservatives)	Phenanthrene, β-naphthol, preservatives: methylparaben, ethylparaben and propylparaben, thiourea (EOF marker) and vitamin K_1
Applications within pharmaceuticals		
10–100% MeOH	Ammonium acetate, acetic acid	Haloperidol and synthetic putative metabolites, pyrazoloacridine and mifentidine
Mixture of MeOH and H_2O	Ammonium acetate, acetic acid	Haloperidol, cimetropium and mifentidine
MeOH	5 mmol L^{-1} ammonium acetate, 100 mmol L^{-1} acetic acid	Haloperidol and its synthetic putative metabolites, pyrazoloacridine and its synthetic putative metabolites, mifentidine and its synthetic putative metabolites
MeOH and mixture of MeOH and MeCN	Ammonium acetate, tetrabutylammonium bromide, tetrabutylammonium hydrogen sulfate and tetrapentylammonium bromide	Tamoxifen and four phase I metabolites
MeOH, MeCN, mixture of MeOH and MeCN, formamide, NMF, DMF, DMA, DMSO	25 mmol L^{-1} ammonium acetate, 0–1 mol L^{-1} acetic acid or 100 mmol L^{-1} sodium acetate Application: 25 mmol L^{-1} ammonium acetate, 1 mol L^{-1} acetic acid in MeCN	Imipramine, di-desmethylimipramine, desmethylimipramine, methylimipramine and imipramine-*N*-oxide. Maprotiline, amitriptyline, litracene, protriptyline and nortriptyline. Application: imipramine *N*-oxide and impurities
MeOH : MeCN : DMF (45 : 49 : 6 v/v/v)	25 mmol L^{-1} ammonium acetate, 10 mmol L^{-1} citric acid and 118 mmol L^{-1} methanesulfonic acid	Tetracycline and three degradation products. Tetracycline, oxytetracycline, doxycycline, desmethyltetracycline and chlortetracycline
MeOH : MeCN (1 : 1 v/v)	20 mmol L^{-1} ammonium acetate, 1 mol L^{-1} acetic acid	Morphine analogues, antihistamines, antipsychotics and stimulants
Formamide, NMF or DMF	Citric acid or acetic acid mixed with Tris. Chiral selectors: β-CD, γ-CD and derivatized β-CD. Addition of long chain alkyl ammonium salts investigated	Racemic mixtures of chlorphedianol, chlorcyclizine, ethopropazine, mianserin, nefopam, primaquine, propiomezine, trihexyphenidyl, trimeprazine, trimipramine and thioridazine
NMF, formamide and mixtures of both	25–200 mmol L^{-1} β-CD, 10 mmol L^{-1} NaCl	Dansylated amino acids
NMF	5–100 mmol L^{-1} β-CD and 10 mmol L^{-1} NaCl	Dansylated amino acids
MeOH	Ammonium acetate, acetic acid, quinine	*N*-3,5-dinitrobenzylated amino acids, ($\pm$)-1,1′-binaphthyl-2,2′-diyl hydrogen phosphate and *N*-[1-(1-naphthyl)ethyl]phthalomic acid
MeCN	($\pm$)-Camphorsulfonic acid potassium or sodium salt, 1 mol L^{-1} acetic acid 0.2 mol L^{-1} Tween 20	Atenolol, bisoprolol, bunitrolol, metroprolol, pindolol, propranolol, salbutamol, ephedrine, epinephrine, cisapride and synthetic impurities
Formamide	Tetra-*n*-butylammonium perchlorate. Chiral selector: (+)-18-crown-6-tetracarboxylic acid	1-Naphthylethylamine, 1-phenylethylamine, phenylalanine, DOPA, tryptophan, norephedrine, noradrenaline and 2-amino-1,2-diphenylethanol

Table 3 *Continued*

Solvents	*Electrolytes*	*Analytes*
Formamide, NMF, DMF, DMA, DMSO, MeOH, MeCN and mixtures of MeOH and MeCN	25 mmol L^{-1} ammonium acetate, 1 mol L^{-1} acetic acid	Morphine, codeine, normorphine, thebaine, noscapine and papaverine. Application: morphine in opium tincture
MeOH : MeCN (75 : 25)	25 mmol L^{-1} ammonium acetate, 1 mol L^{-1} acetic acid	Morphine
NMF	500 mmol L^{-1} magnesium acetate tetrahydrate	Oxytetracycline in an ointment
Mixtures of MeOH and MeCN	Ammonium acetate, ammonium chloride, acetic acid, trifluoroacetic acid, formic acid, methane sulfonic acid	*Cis-trans* (*Z-E*) isomers of chlorprothixene, thiothixene, clopenthixol, flupenthixol, flupenthixol decanoate, clomiphene and diastereomers: L-Ala-L-Phe, L-Ala-D-Phe; quinine, quinidine, cinchonine and cinchonidine
Mixtures of MeOH and MeCN	Sodium acetate	A range of penicillins, cephalosporins and nonsteroidal anti-inflammatory drugs
MeOH	20 mmol L^{-1} CAPS and 0–40 mmol L^{-1} Brij 35	Mesoporphyrin, coporphyrin, pentaporphyrin, hexacarboxylporphyrin, heptacarboxylporphyrin and uroporphyrin
Applications within biological fluids		
10–100% MeOH in H_2O	20 mmol L^{-1} ammonium acetate, 1% acetic acid	Pyrazoloacridine, two metabolites and a synthetic degradation product in urine
NMF	500 mmol L^{-1} magnesium acetate tetrahydrate	Tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), demeclocycline, 4-epitetracycline, anhydrotetracycline, 4-epianhydrotetracycline and desmethyltetracycline. TC, OTC and CTC in cow milk and human plasma
MeOH	5 mmol L^{-1} ammonium acetate, 100 mmol L^{-1} acetic acid	Mifentidine and three metabolites in rat liver homogenate
MeOH : MeCN (1 : 1 v/v)	50 mmol L^{-1} ammonium acetate, 159 mmol L^{-1} sodium acetate and 0.002% (w/v) hexadimethrine bromide	Acetylsalicylic acid and three metabolites: salicylic acid, salicyluric acid and gentisic acid in plasma and urine

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NACE provides high separation power of very similar substances without using additives like surfactants or cyclodextrines. In **Figures 1** and **2** the separation of very similar substances using NACE are compared to separation in an aqueous CE and a MEKC system. As seen in Figure 2, even substances expected to have identical mass over charge may be separated in a short time compared to the aqueous systems. **Figure 3** shows the separation of *cis-trans* isomers and diastereoisomers. These isomers are also expected to have the same mass over charge ratio. The use of NACE in the analysis of food, pharmaceuticals and biological fluids has been reviewed by Bjørnsdottir and co-workers and in **Table 3** an overview of applications is given. An important practical consequence of using a NACE separation medium is that the organic phases resulting from either simple extractions or from eluents from solid-phase extractions can be injected directly into the system, thereby saving time.

Furthermore, some NA solvents seem promising for CE-MS experiments due to the volatility of the solvents and the relatively low current generated in the organic solvents. The low current is comparable to the current generated in electrospray MS interfaces and therefore the stability of online CE-MS is optimized.

Two questions are often raised in connection with practical work with NACE. How important is it that the electrophoresis medium is really nonaqueous? This is not crucial. A content of water up to 1% will not influence the separation efficiency and selectivity significantly. Is it possible to perform quantitative analysis using NACE? Yes, if steps against evaporation are taken when volatile solvent are used, the reliability of the methods is comparable to that of aqueous systems (**Table 4**). A number of applications including validated quantitative methods are given in Table 4.

Table 4 Validation data from quantitative NACE analysis of food, pharmaceuticals and biological fluids

Analytes	*Linearity* (r^2)	*Repeatability of inj.* (*%RSD*)	*LOD*	*Accuracy*
Free saturated long chain fatty acids (*n*-C_{14}-*n*-C_{26})	0.994 (n_{16}-n_{20}) 0.985 (n_{22}-n_{26})	Inter-day: 2.1–39% ($n = 6$) at three concentration levels	0.025 mmol L^{-1}	nd
Tetracyclines in plasma and milk	0.999	Inter-day repeatability of the method: 3.6–10.2% ($n = 6$) at three concentration levels	25 ng mL^{-1} of TC, OTC or CTC	97.2% Oxytetracycline ($n = 6$, %RSD = 4.2%) and 63.3% ($n = 6$, %RSD = 3.6%) at two conc. in milk
Vitamin K_1, propylparaben and methylparaben	0.993	~ 3% ($n = 6$) at three concentration levels for all three analytes	nd	98% Vitamin K_1 ($n = 6$, %RSD = 4.95%) 96% Propylparaben ($n = 6$, %RSD = 4.45%) 82% Methylparaben ($n = 6$, %RSD = 2.26%)
Tetracycline and three degradation products	0.993–0.998	Inter-day: 3.4–13% (five concentration levels)	nd	nd
Oxytetracycline in an ointment	> 0.999	Inter-day: 2.8–4.4% for peak area ($n = 6$) at three conc. levels. For migration time: > 0.8% within day ($n = 8$) and < 3.3% in-between days ($n = 6$)	nd	96.1–97.3% Oxytetracycline at three concentration levels ($n = 6$)
Morphine in pharmaceutical products	> 0.999	Inter-day: 2.0% ($n = 6$)	0.2 μg mL^{-1}	100.7–101.2% (three concentrations)
Acetylsalicylic acid and three metabolites: salicylic acid, salicyluric acid and gentisic acid in plasma and urine	Good in the range: 5–500 μg mL^{-1}	Inter-day: plasma: 0.8–5.0% ($n = 6$), urine: 1.0–5.4% ($n = 6$) at three conc. levels	LOQ : 5 μg mL^{-1} in plasma and 25 μg mL^{-1} in urine	Plasma: 65–99%, urine: 75–97%

nd, not determined; LOQ, limit of quantitation. Reproduced with permission from Bjørnsdottir *et al.* (1998) *Electrophoresis* 19: 2179.

Concluding Remarks

The primary advantages of using nonaqueous media for CE may be outlined in four statements:

1. The separation selectivity is improved by using neat organic solvents and the selectivity can easily be altered by changing the nature of the organic solvent or using mixtures of organic solvents.
2. Analysis of hydrophobic compounds is facilitated as their solubility is higher in organic solvents than in aqueous media.
3. Sample preparation is facilitated as extracts obtained with organic solvents may be injected directly into the nonaqueous system (e.g. the eluate from a solid-phase extraction cartridge, when using MeOH or MeCN as the eluent, may be used for CE without further treatment).
4. The relatively low current generated in organic solvents combined with the volatility of the solvents seems to be promising for CE-MS experiments.

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