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Review

Determination of penicillins in milk of animal origin by capillary electrophoresis: Is sample treatment the bottleneck for routine laboratories?

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ABSTRACT

Capillary electrophoresis (CE) is increasingly being used not only for research purposes but also for routine analyses. The latter, however, are especially difficult when the analytes are present at very low concentrations in complex food samples (e.g. penicillins in milk of animal origin). No study of the difficulties encountered in daily practice in sample treatments for the determination of penicillins (PENs) in milk by CE has to our knowledge been reported. Rather than reviewing the main uses of CE for determining PENs in different types of samples, this paper focuses on the weaknesses of available methods for this purpose, which originate in sample treatment rather than in a lack of robustness of the CE technique. Some problems which, based on our own experience, often confront sample treatment and method development in this context are discussed here. Clearly, the greatest source of error in this context is sample processing, which must provide optimal extraction and preconcentration of analytes, and extracts compatible with the separation technique to be used. In this respect, using time-consuming procedures can cause the loss of variable amounts of analytes in different steps. Interestingly, dramatically simplifying the sample preparation process can detract from sensitivity but lead to increased recoveries. As with any methodological development in routine analysis, acceptable results can only be obtained by considering all potentially influential factors.

Contents

1. Introduction

The accurate determination of antibiotics and their metabolites in food samples is critical not only for their quality control, but also to assure public health. In fact, these substances can cause the development of antibiotic-resistant bacteria—which are more difficult to destroy than the original strains—and allergic reactions or be directly toxic [1]. Although, analytically, antibiotics are usually separated by HPLC, capillary electrophoresis (CE) is being increasingly used for this purpose as confirmed by the more than 1200 papers on this topic published in recent years and several state-of-the-art reviews on the use of CE with antibiotics [1,2].

The most important group of antibiotics for human and veterinary medicine is that of β-lactams, which include penicillins

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(PENs) and cephalosporins, and have been widely used as antimicrobial drugs for more than 80 years [3]. The main use of these antibiotics in the dairy industry is to destroy the pathogens behind mastitis, a disease which causes considerable economic losses [4]. Penicillins account for more than one-third of the total antibiotic production [5] and their widespread use has raised the need for tighter controls. To ensure human food safety, many countries including the United States and those of the European Union (EU) have set definitive maximum residue limits (MRLs) for potentially toxic substances in food products. A need for analytical methods allowing the presence of such substances at levels below their MRLs to be detected therefore obviously exists.

A large number of analytical methods for determining and screening PENs have been developed lately [6]. An interesting review of the monitoring of PENs in food samples by CE describing the potential of the electrophoretic technique for detection and quantitation of PENs was published in 2009 [7]. Few routine applications of CE to real food samples, however, to date have been developed owing to (a) the very small sample volumes used in CE (a few nanoliters), which can have an adverse impact on precision; (b) the low sensitivity of the technique, which is a result of the low volume loadability of capillaries during continuous detection [8]; and (c) incompatibility between some samples and CE methods [9]. These shortcomings have been circumvented by developing new approaches to improving sensitivity, selectivity and robustness in CE (see Fig. 1).

CE is known to have limited sensitivity when used with the UV technique owing to the short optical path length available with in-capillary detection. This has promoted the use of alternative techniques such as laser induced fluorescence, electrochemical, chemiluminescence, electrochemiluminescence and mass spectrometry (MS) detection, which are all more sensitive than classical UV–vis detection. Other, novel techniques including contactless conductivity detection (C^4D) and potential gradient detection (PGD) have also been used for this purpose. Also, in-chip CE has attracted much interest in recent years; for example, chip-based microfluidic systems have been used to determine antibiotics [1]. In any case, PENs are most often determined with a UV or MS detector, which are the best suited to their structure and chemical properties.

Although a number of interesting methods testifying to the analytical usefulness of CE for determining PENs currently exist, few—only five—have been used to extract these analytes from milk samples. This may have contributed to the little acceptance of CE for routine food analyses involving the determination of antibiotics.

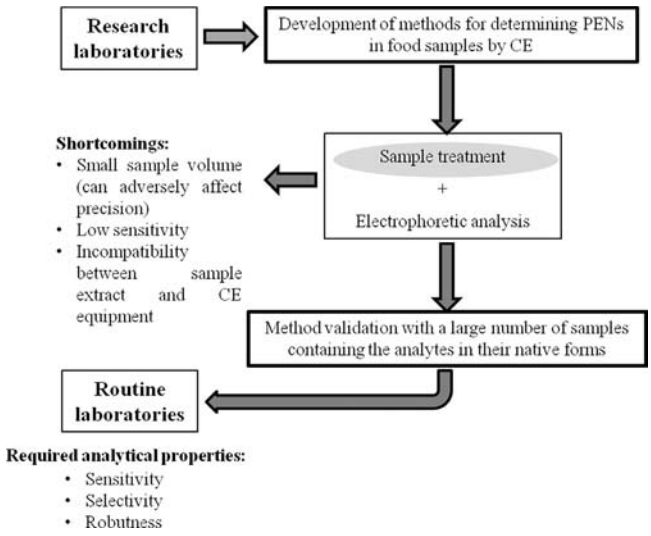

Fig. 1. Shortcomings of sample treatment and required analytical properties for the routine CE analysis of PENs in food samples.

The main difficulty in determining PENs in complex samples such as milk lies in their extraction from the matrix. This step can be the bottleneck of routine analytical methods. Several sample pretreatment steps are required in most cases to extract and preconcentrate the analytes. In fact, some food matrix components such as saline constituents, macromolecules and other major compounds can disturb CE separations. In addition, particulate matter can easily clog a CE system [10]. For these reasons, food samples often require especially complex treatments prior to analysis by CE.

This paper is not a mere review of CE methods for determining PENs in milk; rather, it primarily aims at highlighting the weaknesses of existing methods for this purpose, the greatest of which is sample treatment rather than the characteristics of the CE technique (e.g. its robustness).

2. CE methodologies for the determination of PENs

Research groups worldwide have developed a number methods for determining PENs in food samples. Such methods differ in accuracy, expeditiousness and cost. Most, however, fall into one of these four categories: (a) microbiological methods based on bacterial growth inhibition, (b) biosensing methods; (c) immunochemical methods; and (d) chromatographic or electrophoretic methods. The advantages and drawbacks of these methods, and specific aspects of the determination of PENs with them, are discussed elsewhere [11].

The analytical methods for determining PENs endorsed by the EU (Commission Decision 2002/657/EC) are based on chromatographic techniques and/or analytical molecular spectrometry. However, the EU has stated that regulatory laboratories must find the optimum analytical techniques for determining pharmacological substances, so other methods are expected to be adopted in the future if they prove suitable for the intended purpose [12].

Although PENs are usually separated by HPLC for their subsequent determination, CE is being increasingly used for this purpose by virtue of its high efficiency and simplicity, short analysis times and low consumption of samples and reagents. Also, CE is being increasingly used in routine pharmaceutical and clinical analyses on the grounds of its acceptable analytical performance and good quantitative results. The determination of PENs by CE can be approached in two may ways, namely: (a) by capillary zone electrophoresis (CZE), where a separation buffer with or without additives is used to determine ionic antibiotics by their differences in electrophoretic mobility; and (b) by micellar electrokinetic chromatography (MEKC), where a micellar system (usually a surfactant at a concentration exceeding its critical micelle concentration) is added to the separation buffer to effect the separation of neutral and/or ionic antibiotics by generating a pseudostationary phase for the analytes to partition [13]. CZE (46%) and MEKC (36%) are the preferred separation modes for PENs, but microemulsion electrokinetic capillary chromatography (MEEKC) (11%), cyclodextrin electrokinetic chromatography (CD-EKC) (3.5%) and nonaqueous capillary electrophoresis (NACE) (3.5%) have also been used for this purpose.

Several methods for determining PEN residues by CE have been reported in recent years, [14–39]. Table 1 lists them in chronological order from the most recent to the oldest and shows their experimental conditions (background electrolyte composition, capillary conditioning, temperature, injection pressure and time, voltage, detection system, analysis time and CE instrument used). As can be seen, most PENs were separated by using a borate and/or

Table 1
Summary of existing CE methodologies for the determination of PENs.

Analyte(s)	BGE composition		Pre-conditioning	Post- conditioning	Temperature (°C)	Injection (pressure/ time)	Voltage (kV)	Detector	Analysis time (min)	CE instrument	Ref.
	Buffer	pH									
OXA, PEN V, PEN G, NAF, AMP and AMX	5% SDS, 80% 1-butanol, 15% sodium acetate	8.0	1 min 0.1 M NaOH, 2 min water and 5 min running buffer	n.s.	37.5	50 mbar/3 s	−29	UV–Vis	9	HP^{3D} CE system (Agilent Technologies)	[14]
6-APA, PEN G, AMP and AMX	40 mM potassium dihydrogen phosphate, 20 mM borax solution	7.8	n.s.	n.s.	30	n.s.	28	n.s.	4.5	HP^{3D} CE system (Agilent Technologies)	[15]
NAF, DCLX, CLX, OXA, AMP, PEN G, AMX, PEN V and PIP	60 mM ammonium acetate	6.0	3 min water, 3 min 0.1 M NaOH, 3 min water, and 5 min running buffer (N_2 pressure, 7 bar)	1 min running buffer (N_2 pressure, 7 bar)	30	50 mbar/ 80 s	30	Tandem MS (MS/ MS)	n.s.	HP^{3D} CE system (Agilent Technologies)	[16]
NAF, DCLX, AMP, OXA, PEN V, CLX, PEN G, and AMX	50 mM phosphate 89.27%, SDS 2.21%, 2-propanol 7.71%, propylene glycol monomethyl ether acetate 0.81%	2.0	5 min 0.1 M NaOH and 5 min running buffer	n.s.	30	50 mbar/3 s	−20	UV–Vis	7	HP^{3D} CE system (Agilent Technologies)	[18]
PEN G	30 mM sodium tetraborate	9.2	10 min 100 mM NaOH 5 min water and 10 min running buffer	5 min running buffer	20	1 psi/1–5 s	15	DAD	30	P/ACE MDQ CE system (Beckman-Coulter)	[19]
AMX, AMP, OXA, and PEN V	60 mM ammonium acetate with 10% of methanol (MeOH)	8.0	10 min 0.1 M NaOH and 10 min running buffer (20 psi)	n.s.	20	1 psi/10 s	25	MS	25	P/ACE MDQ CE system (Beckman-Coulter)	[20]
NAF, CLX, OXA, DCLX, AMP, AMX, and PEN G	175 mM Tris buffer with 20% ethanol	8.0	3 min 0.1 M NaOH, 3 min, water and 5 min running buffer (N_2 pressure, 7 bar)	1 min 0.1 M NaOH, 1 min water and 2 min running buffer (N_2 pressure, 7 bar)	30	7 bar/1 min (LVSS)	−20 and 25	DAD	30	HP^{3D} CE system (Agilent Technologies)	[21]
AMP, AMX, CLX, PEN G, tetracycline and chloramphenicol	2.7 mM potassium dihydrogen-phosphate, 4.3 mM sodium tetraborate	8.0	n.s.	n.s.	25	0.5 psi/3 s	18	DAD	15	P/ACE MDQ CE system (Beckman-Coulter)	[22]
CLX, DCLX, OXA, PEN G, PEN V, AMP, NAF, PIP, and AMX	26 mM sodium tetraborate, 100 mM SDS	8.5	2 min 0.1 M NaOH, 2 min H_2O Milli-Q and 2 min running buffer	n.s.	30	50 mbar/5 s	20	DAD	22	HP^{3D} capillary electrophoresis system (Agilent Technologies)	[23]
AMX, DCLX, NAF, PEN V, PEN G, OXA, CLX and AMP	0.5% Ethyl acetate, 1.2% 1-butanol, 2% Brij 35, 10% 2-butanol, 86.3% 10 mM borate	10.0	8 min 0.1 M NaOH, 8 min, water and 10 min microemulsion solution	2 min microemulsion solution	25	50 mbar/5 s	10	DAD	12	HP^{3D} CE system (Agilent Technologies)	[24]
PEN V and related substances	Phosphate–borate buffer with 69 mM SDS and 12.5 mM pentanesulfonic acid sodium salt	6.3	n.s.	n.s.	25	10 s	15	UV	n.s.	Waters Quanta 4000 CE system	[25]
PEN V, AMX, DCLX, NAF, PEN G, OXA, CLX and AMP	20 mM sodium tetraborate, 60 mM SDS	8.0	2 min 0.1 M NaOH, 2 min, water and 2 min running buffer	2 min water	25	50 mbar/ 10 s	15	DAD	17	HP^{3D} CE system (Agilent Technologies)	[26]
AMP	40 mM phosphate–borate buffer, 75 mM SDS	7.5	1 min water, 1 min 0.1 M NaOH, 1 min water, and 5 min running buffer	n.s.	25	0.5 psi/6 s	18	DAD	n.s.	P/ACE MDQ CE system (Beckman-Coulter)	[27]
AMX, AMP, PEN G sodium salt, PEN G-procaine salt, PEN G-benzathine salt, OXA, PEN V and CLX	40 mM sodium tetraborate, 100 mM SDS	8.5	2 min 0.1 M NaOH, 2 min Milli-Q H_2O and 2 min running buffer	n.s.	20	10 s	10	DAD	33	P/ACE MDQ CE system (Beckman-Coulter)	[28]
Benzylpenicillin, procaine, benzathine and clemizole	3.12 g/L disodium hydrogen phosphate, 7.64 g/L sodium tetraborate, 14.4 g/L SDS	8.7	n.s.	n.s.	25	10 s	18	UV	15	Waters Quanta 4000 CE system	[29]
Procaine, dihydrostreptomycin and PEN G	80 mM sodium tetraborate decahydrate	8.0	5 min 0.1 M KOH, 5 min water and 10 min running buffer	n.s.	35	10 s	15	UV	10	Waters Quanta 4000 CE system	[30]

Table 1 (*continued*)

Analyte(s)	BGE composition		Pre-conditioning	Post- conditioning	Temperature (°C)	Injection (pressure/ time)	Voltage (kV)	Detector	Analysis time (min)	CE instrument	Ref.
	Buffer	pH									
OXA, CLX and DCLX	50 mM Phosphoric acid, 5.2 mM 2-hydroxypropyl-beta-cyclodextrin	3.6	n.s.	2 min 0.2 M NaOH, 2 min water, 5 min 0.2 M HCl, 5 min running buffer	25	3.0 psi/50 s	−30	DAD	19	P/ACE MDQ CE system (Beckman-Coulter)	[31]
PEN V and related substances	20 mM ammonium acetate, 20 mM ammonium acetate in 60/40 v/v ACN/MeOH	6.5	n.s.	n.s.	25	50 mbar/3 s	−20	UV and ESI-MS	n.s.	HP^{3D} CE system (Agilent Technologies)	[32]
PEN V, clofibric acid, naproxen, bezafibrate, carbamazepine, diclofenac, ibuprofen, mefenamic acid and paracetamol	20 mM ammonium acetate	5.1	n.s.	3 min running buffer	n.s.	5 kPa/ 0.3 min	20	MS	20	Crystal 310 CE instrument (Thermo CE)	[33]
AMX	20 mM sodium tetraborate	9.0	15 min 0.1 M NaOH, 15 min Milli-Q H_2O and 10 min running buffer	2 min water and 3 min running buffer	30	100 mbar/ 1.8 s	15	UV	12	Prince CE System (Lauer, Emmen, Holland)	[34]
AMX and potential impurities	70 mM sodium dihydrogen phosphate, 125 mM SDS 5% ACN	6.0	5 min running buffer	n.s.	25	4 s	15	UV	20	Spectraphoresis 500 Equipment (Thermo, USA)	[35]
PEN G, 6-APA and phenyl acetic acid	30 mM Tetraborate	9.2	n.s.	n.s.	30	12.7 cm Hg/ 1 s	15	UV	5	Model 270 A CE system (Applied Biosystems)	[36]
PEN V and related substances	40 mM sodium dihydrogen phosphate, 100 mM SDS	7.0	5 min running buffer	n.s.	25	5170 Pa/5–20 s	15	UV	25	Spectraphoresis 1000 (Thermo, USA)	[37]
OXA, AMP, PIP, PEN G, PEN V, CLX, DCLX, cephapirin and NAF	20 mM Sodium tetraborate, 75 mM SDS	8.5	n.s.	n.s.	25	50 mbar/ 3.6 s	15	UV	20	Crystal 310 CE instrument (Thermo CE)	[38]
PEN G	10 mM sodium dihydrogen phosphate, 6 mM sodium tetraborate	9.0	n.s.	n.s.	n.s.	10 s	30	UV	10	n.s.	[39]

OXA: oxacillin; PEN V: penicillin V; PEN G: penicillin G; NAF: nafcillin; AMP: ampicillin; AMX: amoxicillin; 6-APA: 6-amino penicillanic acid; DCLX: dicloxacillin; CLX: cloxacillin; PIP: piperacillin; SDS: sodium dodecyl sulfate; ACN: acetonitrile; n.s.: not stated.

phosphate buffer at a variable pH. Twenty-five different methodologies for separating PENs, half of which use sodium dodecyl sulfate (SDS) micelles in the buffer solutions, have to date been reported. Most of their descriptions include the capillary flushing conditions used before and after electrophoretic analysis because they are thought to influence the accuracy of the results. As can also be seen from Table 1, UV detection was the most popular choice for the determination of PENs by CE, although MS was also used in many cases. Despite of the variety of buffers used, no critical assessment of their effectiveness has so far been published to help others select the most suitable choice for specific separation of PENs.

Table 2 summarizes the applications of CE based methods for PENs in the pharmaceutical, environmental, food and clinical fields, among others. The table shows the CE separation mode used and the sample preparation requirements in each case. The listed references were located by using the following search string on the ISI Web of Knowledge database: "capillary electrophoresis" or "CE" or "micellar electrokinetic capillary chromatography" or "MEKC" or "electrokinetic capillary chromatography" or "EKC" and "β-lactam" or "penicillins". As can be seen, the largest number of uses correspond to pharmaceutical applications. This is consistent with some studies indicating that CE is a well-established, frequently used technique in the pharmaceutical industry. In this field, sample pretreatment is usually uncomplicated, precision good and sample throughput high [40]. Note that the most samples used to determine PENs by CE were pharmaceutical preparations, drugs or other commercial pharmaceutical products, followed by milk samples (see Fig. 2).

3. Analytical methodologies for determining PENs in milk samples by CE

CE methodologies have been used to determine various PENs in food samples such as water, milk and animal tissue (see Table 2). To our knowledge, however, CE has not been extensively used to determine PENs in milk samples [15,17,21,22,31], possibly because of the difficulties resulting from the high complexity of this biological matrix and the typically low concentrations of these compounds in milk samples. A large sample size is usually needed to obtain the required sensitivity level, which is no problem with milk samples. With trace and ultra-trace contaminants, an increased method selectivity can help reduce potential matrix interferences and obtain the sensitivity required to determine some analytes.

Only five CE methodologies for the determination of PENs in milk have to date been reported. As can be seen in Table 2, they use CZE, MEKC or CD-EKC for separation. Tian et al. [15] developed a CE method for the simultaneous determination of penicillin intermediates and PENs including 6-amino-penicillanic acid (6-APA), ampicillin (AMP), amoxicillin (AMX) and penicillin G (PEN G) in milk. The four PENs were baseline separated within 4.5 min by using a running buffer consisting of 40 mM potassium dihydrogen phosphate and 20 mM borax at pH 7.8. The average recoveries obtained at three different fortification levels fell in the range 85–97% and relative standard deviations (RSDs) were acceptable: 1–9%.

A different method was used for the simultaneous determination of seven PENs in fortified milk samples in less than 30 min by using 175 mM Tris at pH 8.0 containing 20% ethanol and UV detection at 220 nm. The sensitivity was improved by using SPE in combination with a capillary stacking preconcentration methodology such as large volume sample stacking (LVSS) injection. This protocol afforded limits of detection (LODs) ranging from 2 to 10 $\mu g\ L^{-1}$, which are below the MRLs set by the EU directive for milk, and acceptable recoveries from bovine raw milk (86–93%), bovine skimmed milk (88–93%) and goat raw milk (87–91%) [21].

Finally, a CZE method was proposed for the simultaneous detection of AMX, AMP, cloxacillin (CLX) and PEN G in spiked milk samples. The CE analysis time was 15 min. Quantifying AMX was impossible owing to its poor recovery, which was mainly a result of inefficient SPE extraction. Recoveries were largely influenced by the SPE cartridges used, which were unsuitable for extracting the more polar antibiotics such as AMX. In any case, the average of recovery for the other antibiotics as a whole exceeded 72%. LODs were 0.48–1.09 $\mu g\ mL^{-1}$ and LOQs 1.59–3.64 $\mu g\ mL^{-1}$ [22].

The MEKC mode was also used to separate AMP, AMX and penicillin V (PEN V) in spiked milk samples by using a phosphate buffer containing SDS. LODs were 0.16–0.20 $mg\ L^{-1}$ and the average recoveries of PENs from milk were over 70% for all analytes except AMX [17]. Because these compounds are neutral or weakly ionic molecules, MEKC is often the CE mode of choice for their separation.

As regards CD-EKC, CDs and various derivatives have been used in CE for the separation of isoxazolylpenicillins. Thus, Zhu et al. [31] developed a method of this type for the determination of CLX, oxacillin (OXA) and dicloxacillin (DCX) in milk samples. The method involves large-volume sample stacking with an electroosmotic flow (EOF) pump (LVSEP), separation with 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) as a selective complex-forming background electrolyte additive and direct UV detection. The ensuing LOD for all analytes was 2 $\mu g\ L^{-1}$. All milk samples were spiked with the isoxazolylpenicillins.

All above-described studies were conducted on spiked milk samples. Although finding suitable real-life samples is often difficult, it is usually desirable to use the analytes in their native forms to validate new methods. Also, the LODs and LOQs obtained often exceed the MRLs—typically in the ppm region—for the analytes, which testifies to the difficulty of obtaining adequate sensitivity.

3.1. Preparation of milk samples for the determination of PENs

Sample preparation in an analytical process is usually intended to (a) dissolve the analytes in a smaller matrix size; (b) reduce or avoid the use of organic solvents; (c) serve as a generic extraction procedure for multiclass compounds; (d) integrate several preparation steps into one; or (e) automate and/or expedite determinations [41]. The determination of trace analytes such as PENs by CE based analytical techniques usually requires their prior extraction from the matrix and preconcentration [42,43]. Sample treatment and preconcentration are two crucial parts of chemical analysis and, in a sense, have become the bottlenecks of the whole analytical process [43].

Although SPE and liquid–liquid extraction (LLE) continue to be the most widely used extraction and concentration technique, respectively, milk samples often contain a large number of matrix components that may coelute with the analytes and disturb quantitative analyses. A growing search therefore exists for time- and labor-saving sample pretreatment methods to reduce matrix content and enrich samples with the target analytes. Also, the new methods are expected to be more eco-friendly (i.e. to use smaller amounts of solvents and samples) and, ideally, to require as few operations as possible in order to minimize potential errors and shorten analysis times. In this respect, some cleanup/concentration methodologies such as solid-phase microextraction (SPME), stir bar sorptive extraction (SBSE), matrix solid-phase dispersion (MSPD), hollow fiber (HF) extraction, supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), cloud point extraction (CPE), and dispersive liquid–liquid microextraction (DLLME) have proved effective for preconcentration purposes and hence for separation, identification and quantitation by CE [41].

Table 2
Analytical determination of penicillins by CE in various types of matrices.

Field of application	Matrix	Analyte(s)	CE mode	Sample treatment	Ref.
Pharmaceutical	Pharmaceutical preparations, drugs and other commercial pharmaceutical products	NAF, DCLX, AMP, OXA, PEN V, CLX, PEN G, and AMX	MEEKC	Mixing with water and sonication. The resulting clear liquid is filtered and diluted with phosphate buffer at pH 2 or 8	[18]
		CLX, DCLX, OXA, PEN G, PEN V, AMP, NAF, PIP, AMX	MEKC	Dissolution in water with ultrasound and filtering	[23]
		AMX, DCLX, NAF, PEN V, PEN G, OXA, CLX and AMP	MEEKC	Dissolution in water and filtering	[24]
		PEN V and related substances	MEKC	n.s.	[25]
		PEN V, AMX, DCLX, NAF, PEN G, OXA, CLX and AMP	MEKC	n.s.	[26]
		AMP	MEKC	Sonication for 3 min and filtering	[27]
		Benzylpenicillin, procaine, benzathine and clemizole	MEKC	Dissolution in water	[29]
		Procaine, dihydrostreptomycin and PEN G	CZE	Dissolution in water	[30]
		AMX and potential impurities	MEKC	n.s.	[35]
		PEN G, 6-APA and phenyl acetic acid	CZE	n.s.	[36]
		PEN V and related substances	MEKC	n.s.	[37]
		OXA, AMP, PIP, PEN G, PEN V, CLX, DCLX, cephapirin and NAF	MEKC	n.s.	[38]
Environmental /Food	Water (waste, well, river, surface and potable)	NAF, DCLX, CLX, OXA, AMP, PEN G, AMX, PEN V and PIP	CZE	Extraction with ACN, preconcentration and cleanup with SPE (HLB and Alumina N cartridge)	[16]
		AMX, AMP, PEN G-sodium salt, PEN G-procaine salt, PEN G-benzathine salt, OXA, PEN V, and CLX	MEKC	Filtration	[28]
		PEN V, clofibric acid, naproxen, bezafibrate, carbamazepine, diclofenac, ibuprofen, mefenamic acid and paracetamol	CZE	LLE and SPE	[33]
Food	Milk	6-APA, AMX, AMP and PEN G	CZE	n.s.	[15]
		AMP, AMX, PEN V and cephalexin	MEKC	Protein precipitation and SPE	[17]
		NAF, CLX, OXA, DCLX, AMP, AMX, and PEN G	CZE	Solvent extraction with ACN and SPE (HLB and Alumina N cartridge) for cleanup and preconcentration, in combination with LVSS (in-line preconcentration)	[21]
		AMP, AMX, CLX, PEN G, tetracycline and chloramphenicol	CZE	Protein precipitation with TCA and SPE (C18)	[22]
		OXA, CLX and DCLX	CD-EKC	Extraction with ethyl acetate and large-volume stacking using an electroosmotic flow pump (LVSEP)	[31]
	Animal tissue (porcine organs, chicken muscle, meat and fish)	OXA, PEN V, PEN G, NAF, AMP and AMX	MEEKC	Extraction with ACN and *n*-hexane. SPE with C18.	[14]
		NAF, DCLX, CLX, OXA, AMP, PEN G, AMX, PEN V and PIP	CZE	Extraction with can, and preconcentration and cleanup with SPE (HLB and Alumina N cartridge)	[16]
		AMX, AMP, OXA, and PEN V	CZE	ACN (extraction and protein precipitation) and cleanup with SPE (C18)	[20]
Clinical	Biological fluids (urine, blood, plasma, gastric contents and amniotic fluid)	PEN G	CZE	SPE (C18)	[19]
		AMX	CZE	SPE (C18)	[34]
		PEN G	CZE	Purification by centrifugation and DEAE cellulose treatment of stomach contents (diluted with pH 9 phosphate–borate buffer)	[39]
Other	Fermentation broth	PEN V and related substances	CZE/ NACE	n.s.	[32]

NAF: nafcillin; DCLX: dicloxacillin; AMP: ampicillin; OXA: oxacillin; PEN V: penicillin V; CLX: cloxacillin; PEN G: penicillin G; AMX: amoxicillin; PIP: piperacillin; 6-APA: 6-amino penicillanic acid; n.s.: not stated.

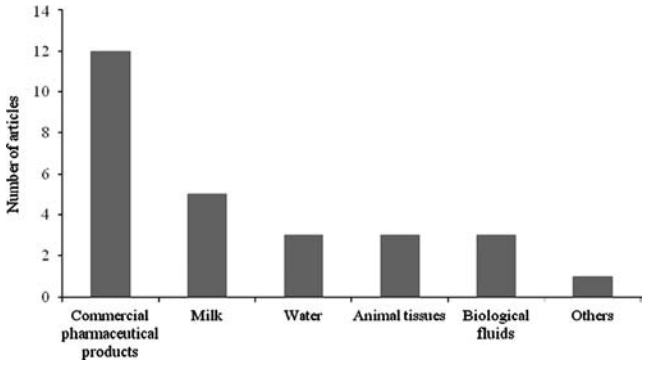

Fig. 2. Number of articles dealing with CE methods for the determination of PENs in various type of matrices until May 2013 as derived from information on the ISI Web of Knowledge database.

A number of procedures have been used for improved preconcentration of the analytes and cleanup of different types of food matrices. The extraction of PENs from milk samples prior to analysis by CE usually includes several steps such as protein precipitation, extraction and preconcentration. Fig. 3 depicts the most common procedures for determining PENs in milk samples by CE. Current methods for pretreating milk samples involve protein precipitation with a reagent such as trichloroacetic acid (TCA) [22] or acetonitrile (ACN) [21]. SPE using C18, alumina N or Oasis HLB polymeric sorbent—which contains lipophilic divinylbenzene units and more hydrophilic N-vinylpyrrolidone units—have been used for further clean up and preconcentration of analytes [17,21,22].

New methodologies have recently been proposed for processing antibiotic-containing milk samples with commercially available molecularly imprinted polymers (MIPs) as SPE sorbents

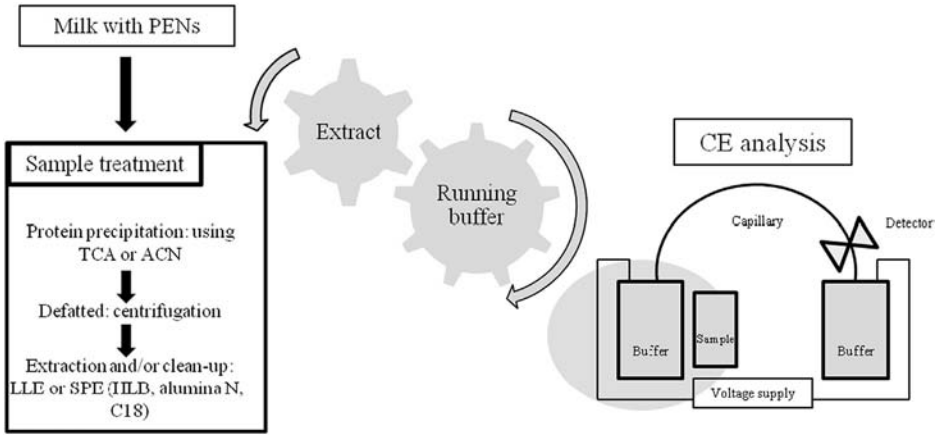

Fig. 3. Compatibility between sample pretreatments and CE analysis.

(MISPE). MIPs are synthetic materials with artificially generated recognition sites capable of specifically capturing target molecules the increased selectivity of which facilitates the easy obtainment of clean sample extracts relative to conventional SPE sorbents. Some such materials have been custom-synthesized in several laboratories. Thus, Quesada-Molina et al. [44] prepared an MIP specific to two cephalosporins (a subclass of β-lactam antibiotics) for use as a template for the imprinted polymer. The MIP exhibited useful cross-selectivity and successfully extracted three structurally related compounds from complex samples such as milk with acceptable recoveries in preliminary tests. The results were evaluated against HPLC with DAD detection.

Other strategies involving QuEChERS—which stands for Quick, Easy, Cheap, Effective, Rugged and Safe—and dispersive extraction by QuEChERS in MSPD format have also been used to treat milk samples containing antibiotics. QuEChERS methodology has some advantages over SPE and other traditional extraction methods such as operational simplicity and effective cleanup of complex samples. The original procedure involves initial SPE of the sample with ACN, followed by liquid–liquid partitioning by addition of anhydrous magnesium sulfate and sodium chloride. Water removal and cleanup are simultaneously achieved on an aliquot of the ACN extract by using dispersive SPE with $MgSO_4$ and a primary or secondary amine sorbent [45]. This methodology has been extensively used to extract pesticide residues from fruits and vegetables [46,47], and also, recently, to determine antibiotic residues in various types of food samples such as animal tissue [48,49], eggs [50] and milk [51,52].

Modified QuEChERS sample preparation procedures have been widely used to date. Thus, a modified MSPD procedure was used for the extraction and cleanup of PENs and amphenicols in milk with a mixture of Strata by Phenomenex and QuEChERS as sorbent [53]. Because milk is a complex matrix requiring sophisticated sample preparation to isolate target analytes, the combination of ultrasonically assisted MSPD and QuEChERS facilitates the preparation of milk samples for HPLC analysis. Moreover, sonication increases recoveries by ensuring efficient contact between the solid and extractant [54]. To the best of our knowledge, QuEChERS methodology has never to date been used to obtain PEN-containing extracts from milk samples for analysis by CE, however.

3.2. Practical considerations on the treatment of milk samples for extraction of antibiotics

Ensuring proper development of the whole analytical process in the CE determination of antibiotics requires that the CE buffer be compatible with the extract provided by the sample pretreatment. The importance of fulfilling this requirement is illustrated by Fig. 3. It should be noted that the sample treatment needed to determine PENs in milk by HPLC will not necessarily be compatible with separation in an electrophoretic system.

Various off-line and in-line preconcentration strategies have been developed with provision for the requirements of the EU Directive for antibiotic MRLs in foods of animal origin including milk, and the typically limited sensitivity of CE methods using UV–vis detection; all have proved effective for determining various analytes at low concentrations.

Off-line SPE, which is probably the most widely used sample pretreatment procedure for preconcentrating analytes prior to CE, requires careful control of all variables potentially influencing sorption and desorption of the analytes in the sorbent. One obvious variable to be considered is the natural pH of the medium containing the analytes; in fact, the extraction process is often optimized by using standard solutions, which are often incompatible with natural samples owing to a marked difference in pH.

Various in-line strategies including LVSS (also called “stacking matrix removal” [21]) and LVSEP [31] have also been used to preconcentrate PENs from milk. Although LVSS is an effective choice for concentrating analytes, it only works with low-conductivity matrices. In fact, high-conductivity matrices such as milk require labor-intensive pretreatment involving several matrix extraction and cleanup steps prior to LVSS-CE. Vera-Candioti et al. [55] found application of LVSS to high-conductivity milk to interfere with matrix removal and preclude its use for preconcentrating antibiotic residues in milk and their subsequent quantitation by CE.

4. Conclusions

CE is a useful, effective alternative to chromatographic methods for monitoring PENs in milk of animal origin. Various CE modes, but especially CZE and MEKC, have been used to determine a great variety of PENs. Although direct UV detection is the most popular choice, the poor sensitivity of CE with this detection mode has required the development of effective strategies to improve the sensitivity of the CE–UV couple. Various methodologies have been proposed for sample cleanup and off-line or in-line preconcentration of analytes. Thus, SPE has been extensively used for off-line preconcentration in this context. New extraction systems for determining PENs such as MISPE and QuEChERS have also been successfully used with a high efficiency; however, QuEChERS methodology has not yet been used to determine PENs in milk by CE. In-line preconcentration procedures (LVSS and LVSEP),

which introduce very large sample volumes to improve sensitivity, have recently started to be used in this area; however, successful implementation of this strategy requires obtaining low conductivity extracts for milk samples to be highly efficiently cleaned up.

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