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Analytical Methods

Method development for sensitive determination of nisin in food products by micellar electrokinetic chromatography

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ABSTRACT

A rapid and sensitive micellar electrokinetic chromatography (MEKC) method has been developed for the identification and quantification of nisin in food products. Factors such as micelle concentration, pH, and concentration of the buffer were investigated in order to determine the optimum conditions for the analysis. Using optimised conditions of a background electrolyte containing 50 mM sodium phosphate, 80 mM sodium dodecyl sulphate (SDS) at pH 3.75 enabled the successful detection of nisin within 6 min. Limits of detection (LOD) and quantification (LOQ) were in the range of 0.3–0.8 and 1.0–2.8 mg L^{-1}, respectively. The calibration curves were linear for nisin concentration over the range of 2–60 mg L^{-1}. The relative standard deviation (RSD) of the peak area ratios and migration times for method repeatability ($n = 3$) were found to be lower than 5% and 1%, respectively. The potential of the proposed method to be used for quantitative determination of nisin concentrations in food materials was demonstrated by spiking food samples including dairy products, salad dressings, alcoholic beverages and canned tomatoes with known concentrations of nisin. Quantitative recoveries ranging from 83% to 104% were obtained in the food matrices.

1. Introduction

Nisin consists of 34 amino acids and belongs to a special group of antimicrobial peptides called bacteriocins and it is further classified as a lantibiotic for having unusual amino acids in its structure such as lanthionines and β-methylanthionines (Cotter, Hill, & Ross, 2005; van Belkum & Stiles, 2000; Willey & van der Donk, 2007). The structure is shown in Fig. 1. The presence of the unusual amino acids provides greater efficiency in binding specific pathogens most often Gram positive bacteria. Nisin also becomes effective against Gram negative bacteria in the presence of chelating agents, sub-lethal heat, osmotic shock and freezing (Delves-Broughton, 2005). Nisin is produced by lactic acid bacteria (LAB), *Lactococcus lactis* (Ayad, Verheul, Wouters, & Smit, 2002; Cheigh & Pyun, 2005; Cintas, Casaus, Herranz, Nes, & Hernàndez, 2001; Cotter et al., 2005; Twomey, Ross, Ryan, Meaney, & Hill, 2002; van Belkum & Stiles, 2000; Willey & van der Donk, 2007). Nisin has biomedical applications. It is used for oral hygiene for treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) (Wang et al., 2005) and enterococcal infections; used for cosmetic deodorants and topical formulations; treatment of peptic ulcer and enterocolitis; and for lung mucus clearing (Cotter et al., 2005). Nisin's most common and important application is its use as a food preservative (Chollet, Sebti, Martial-Gros, & Degraeve, 2008; Delves-Broughton, 2005). Its potential as a promising food preservative has been shown since 1969 when it was declared as a safe, natural food additive by the joint FAO/WHO (1969) expert committee. By 1996, it was allowed as a food additive in more than 50 countries (Delves-Broughton, 2005; Hakovirta, Reunanen, & Saris, 2006). Nisin is highly stable in acidic conditions (pH 3–3.5) and it is insensitive to autoclaving (121 °C for 15 min), allowing for its use in acidic foods that cannot be heat sterilised (Delves-Broughton, 2005). Nisin is suitable for many types of foods from liquid to solid foods, chilled to warm-storage foods, and canned to packaged foods (Thomas, Clarkson, & Delves-Broughton, 2000). In addition, it is used in dairy products, such as milk, processed cheese, cheese spreads, and puddings and also used to preserve salad dressings, vegetables, and alcoholic beverages such as beer (Delves-Broughton, Blackburn, Evans, & Hugenholtz, 1996). However, different regulations exist around the world regarding the levels of nisin allowed in foods (Hakovirta et al., 2006). The activity of nisin decreases during food processing and storage, due to the temperature, pH, and components of food (Delves-Broughton, 2005). As a result, quantification of nisin is vital for monitoring nisin quantities added to foods, and also its stability during the product's shelf life (Hakovirta et al., 2006).

The most popular method for detection and analysis of nisin in food samples is agar diffusion assay where antimicrobial activity is monitored on indicator bacteria strains (Tramer & Fowler, 1964).

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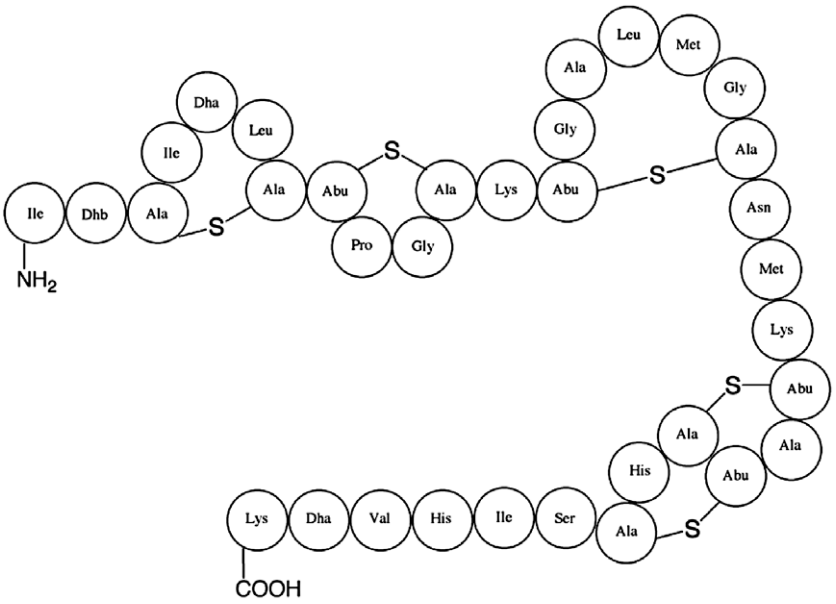

Fig. 1. Primary structure of nisin (M. W. = 3354 Da) showing 34 amino acid residues.

This method has limitations which include the type of test organism used, the presence of interfering substances in fermentation broth or food extracts, interference by the low pH of the samples, agar depth and concentration (Hakovirta et al., 2006; Wolf & Gibbons, 1996). Other analytical methods include immunoassays (Leung, Khadre, Shellhammer, & Yousef, 2002; Nandakumar, Nandakumar, & Mattiasson, 1999; Suárez, Rodríguez, Hernández, & Azcona-Olivera, 1996) and bioassays (Hakovirta et al., 2006; Reunanen & Saris, 2003). The immunoassay methods are generally sensitive to small concentrations of nisin, but they are not totally reliable because of cross-reactions with compounds structurally related to nisin. The drawbacks of the bioassay techniques include longer analysis time, tedious sample preparation and difficulty to process multiple samples at the same time. In addition, bioassays suffer from low specificity, low sensitivity and interference from substances present in food extracts and fermentation broths (Fowler, Jarvis, & Tramer, 1975). Recent enzyme-linked immunosorbent assay (ELISA) methods have also been used for detection of nisin (Falahee, Adams, Dale, & Morris, 1990; Mattiason, Nillson, Berdén, & Håkanson, 1990). The ELISA method has high sensitivity and the precision is usually of the order of a few percent. However, it detects both biologically-active nisin and its non-active degradation molecules; these methods may therefore be considered unsuitable as assay procedures (Delves-Broughton & Friis, 1998). Turbidimetric assay of nisin (Flôres, de Mattos Braga, & Monte Alegre, 2003) has also been done in the past, but it is quite tedious and time consuming.

Liquid chromatography/mass spectrometry (LC/MS) (Zendo, Nakayama, Fujita, & Sonomoto, 2008) has also been used in detecting nisin. This method is highly sensitive, however, larger sample volumes and organic solvents are required for liquid chromatography. In recent years, capillary electrophoresis (CE) has gained a lot of attention due to its high efficiency, fast separation, inexpensive capillaries, small sample size requirements, and low reagent consumption (Baker, 1995; Wehr, Rodríguez-Diaz, & Zhu, 1999). Only one published work has used capillary zone electrophoresis for determining nisin (Rosanno et al., 1998). In that work, a coated capillary was used and laborious extraction procedure was used to extract nisin from milk before analysing it. In this paper, we report a micellar electrokinetic chromatography (MEKC) method for determining nisin in food products and alcoholic beverages. The method described in this paper uses an uncoated capillary for the analysis. In addition, this MEKC method is efficient, sensitive and rapid, and does not involve any complicated extractions.

2. Experimental

2.1. Standards and reagents

Nisin was obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA) as a crude milk culture extract containing 2.5% nisin, NaCl and denatured milk solids. Stock standard solutions of 500 and 100 mg L^{-1} of nisin were prepared by dissolving appropriate amounts of nisin in 10 mM sodium phosphate solution of pH 3.0. Standard solutions of nisin were sonicated for 2 min for dissolution then adjusted to pH 3.0 (±0.01). Sodium dihydrogen phosphate ($NaH_2PO_4{\cdot}H_2O$) and sodium dodecyl sulphate (SDS) were also obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). The background electrolyte (BGE) consisting of phosphate and SDS was prepared by dissolving SDS and sodium dihydrogen phosphate in 18 MΩ water to obtain the desired concentration. Phosphate concentrations were varied from 20 mM to 60 mM (in increments of 10 mM) containing various SDS concentrations of 60 mM–100 mM (in increments of 10 mM). These solutions were used for optimisation studies. Buffers were sonicated and then adjusted to the required pH. The pH of the buffers and stock solutions were adjusted by using 1.0 M NaOH (BDH Chemicals Toronto, ON, Canada), 6 M or 3 M HCl (Sigma–Aldrich, St. Louis, MO, USA) and monitored by Symphony SB90M5 pH meter. Standards and stock solutions were diluted to volume using deionised 18 MΩ water. All reagents were of analytical grade. Stock solutions were stored in plastic bottles at 4 °C. All solutions were filtered through 0.22-μm sterile, Nylon syringe filters prior to use for the CE experiments. The background electrolyte (BGE) was replaced regularly with fresh buffer.

2.2. Sample preparation

Food samples – 2% milk, whipping cream, homogenised milk, salad dressing (Raspberry vinaigrette, and Thousand islands), canned tomatoes, processed cheese, plain yogurt, yogurt drink –

were obtained from a local grocery store. The pre-drop beer was provided by Columbia Brewery (Creston, BC, Canada), and the wine sample was obtained from a local liquor store. Milk samples (5 mL) and the pre-drop beer (5 mL) were filtered using Amicon® Ultra-4 3 kDa Centrifugal Filter Units (Billerica, MA, USA) in a swinging bucket rotor at 4000*g*. Salad dressings (2 mL) were diluted 1:10 with deionised water, homogenised and then filtered with P8-Creped Fisher brand qualitative filter paper. Following that, the Raspberry vinaigrette and Thousand islands salad dressings were filtered through Amicon® Ultra-4 3 kDa and 10 kDa filter units, respectively. Plain yogurt (2 mL) was diluted 1:10 and yogurt drink (3 mL) was diluted 1:5 and both were homogenised and then filtered using Amicon® Ultra-4 5 kDa; processed cheese (1 g) was diluted 1:20, homogenised and then filtered using Amicon® Ultra-4 5 kDa; canned tomatoes (1 g) was diluted 1:5, homogenised before filtering using Amicon® Ultra-4 5 kDa. The red wine (5 mL) was diluted 1:5 and then filtered using Amicon® Ultra-4 5 kDa. All samples were centrifuged at 4000*g* for 10–20 min. After centrifugation, samples were further filtered using 0.22-μm Nylon syringe filters and then spiked with different nisin concentrations to make up a total volume of 300 μL. Calibration standards ranging from 2 mg $L^{-1}$to 60 mg L^{-1} were always prepared the same day as they were run in the instrument. For the phosphate buffer calibration curve, nisin standards were prepared for concentrations of 2, 5, 10, 15, 20, 30, 40, 50, and 60 mg L^{-1}. For the calibration curves in the food samples, nisin standards were prepared for concentrations of 5, 10, 15, 20, 30, 40, 50, and 60 mg L^{-1}.

2.3. Instrumentation and electrophoretic procedure

A Beckman P/ACE™ System MDQ Capillary Electrophoresis unit (Fullerton, CA) equipped with ultraviolet detector was employed for all CE analysis. The analysis was carried out with an uncoated fused silica capillary (50 cm × 50 μm ID), housed in a cartridge configured for UV detection. Nisin was detected at 214 nm using direct absorbance, 20 kV (reversed polarity), and at a constant temperature of 25 °C. The capillary was rinsed with 0.1 M NaOH for 15 min and the run buffer for 15 min at 20 psi everyday prior to use. Before each injection, the capillary was rinsed for 3 min with 0.1 M NaOH, 3 min with deionised water, and finally 3 min with the run buffer. All experiments were performed using the same capillary. Samples were injected at a 5 s interval and a pressure of 0.5 psi (3.45 kPa).

3. Results and discussion

To investigate MEKC as a method for the detection and quantification of nisin, several factors were optimised such as the phosphate concentration, pH, and SDS concentration.

3.1. Optimisation of MEKC conditions

In order to study the ionic strength effect of the buffer due to the phosphate concentration, a constant amount of SDS was added to varying concentrations of the phosphate buffer. A solution containing 80 mM SDS concentration was added while keeping the pH constant at 3.75 and varying the phosphate concentration from 20 mM to 60 mM in increments of 10 mM (Fig. 2A). The analysis time decreased as phosphate concentration increased (Fig. 2A), indicating that on increasing the concentration of the buffer the electroosmotic flow (EOF) increases. The EOF is the movement of the buffer solution inside the capillary when a potential is applied across the capillary. As the concentration of the phosphate is varied, the peak areas of nisin stayed relatively constant with just a small difference in the intensities of the peaks. It can also be seen from Fig. 2A that the migration time between 50 and 60 mM phosphate concentration did not change much and, furthermore, the 50 mM produced the sharpest peak. Thus, the optimum phosphate concentration was chosen to be 50 mM.

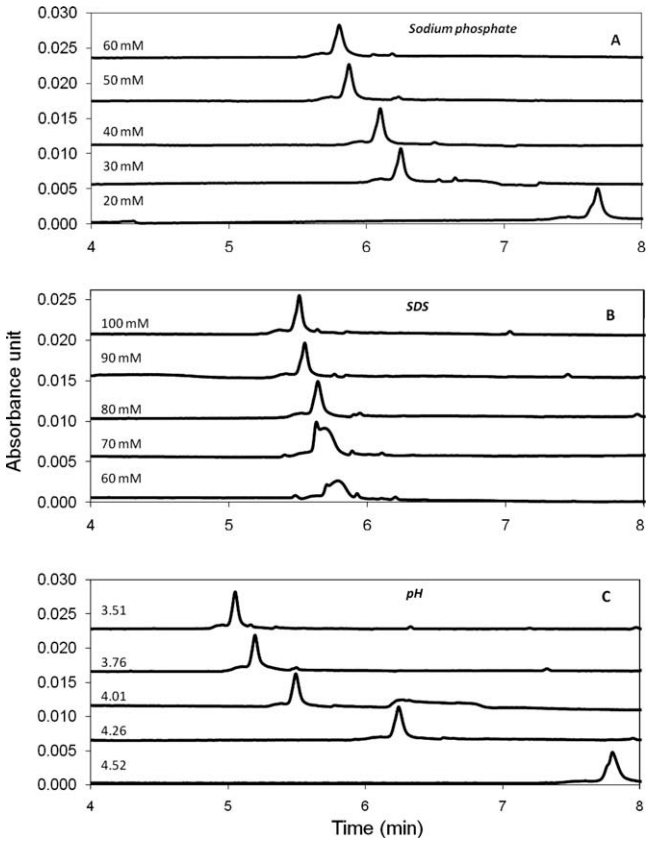

Fig. 2. MEKC optimisations: (A) sodium phosphate optimisation at 80 mM SDS, pH 3.74 ± 0.01 (B) SDS optimisation at 50 mM sodium phosphate, pH 3.75 ± 0.01 (C) pH optimisation using 50 mM sodium phosphate concentration, 80 mM SDS. All optimisations were carried out using reverse polarity and 500 mg L^{-1} nisin standard.

To investigate the effect of SDS concentration on the nisin peak, concentration of SDS was varied from 60 mM to 100 mM (Fig. 2B) at the optimum phosphate concentration of 50 mM and at a pH 3.75 ± 0.01. Changing the SDS concentration did not change the migration time of the nisin peak significantly. However, it can be seen from Fig. 2B that, at the lower SDS concentrations of 60 mM and 70 mM the peaks were broader and had irregular shapes. This could be due to increased adsorption of nisin onto the walls of the capillary. At 80 mM SDS and beyond, the nisin peak became sharper and more symmetrical. Increasing the SDS concentration favours ion-pairing of nisin with SDS to form SDS-micelle. This results in lower tendency for nisin to adsorb onto the capillary walls. Hence, the peaks get sharper as shown in Fig. 2B. Hence, the optimum SDS concentration was chosen to be 80 mM.

The buffer pH mainly influences the strength of the electroosmotic flow (EOF) which is crucial to CE analysis. The pH was optimised by increasing it from ~3 to 4.5 in increments of 0.25 (Fig. 2). Nisin has a net 3+ charge and, thus, being cationic can ion-pair with the anionic SDS to form a nisin–SDS-micelle which is negatively charged. This is what carries the nisin to the detector at the anode. As the pH is increased from ~3 to 4.5 there is a slight increase in the migration time of nisin. This could be due to the fact that as the pH is increased, nisin becomes less cationic and ion-pair less to the SDS. As a result it does not travel fast to the detector at the anode and the migration time gets longer. As can be seen in

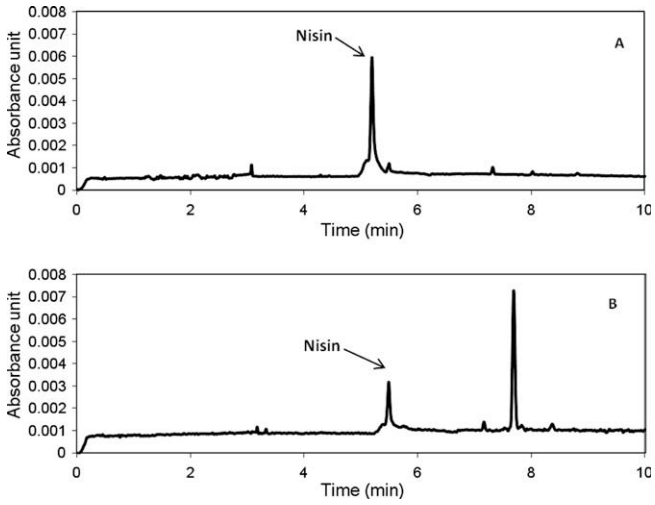

Fig. 3. Comparison between electropherograms of (A) 500 mg L^{-1} nisin in 10 mM sodium phosphate at pH 3.0 ± 0.01 and (B) whipping cream sample spiked with 50 mg L^{-1} nisin standard. Both electropherograms were obtained at optimum buffer conditions: 50 mM sodium phosphate, 80 mM SDS and pH 3.75 ± 0.01.

Fig. 2C, the change in migration time is more noticeable between 4.01 and 4.52, while the migration time hardly changed from pH 3.51 to 3.76. Hence the optimum pH was chosen to be 3.75 ± 0.01. Optimum MEKC conditions obtained were 50 mM sodium phosphate, 80 mM SDS, and pH = 3.75. Fig. 3A shows the electropherogram for 500 mg L^{-1} nisin standard ran using the optimum MEKC conditions with nisin being successfully detected within 6 min.

3.2. Regression equations, detection limits, recoveries, and reproducibility for MEKC

In order to evaluate the validity of these conditions, a calibration curve was generated by running three replicates at each of the nine concentrations of nisin standard (as indicated in Section 2.2) in 10 mM phosphate buffer at pH 3.75. Good linear response was obtained from 2 mg L^{-1} to 60 mg L^{-1} with an R^2 value of 0.993. Three replicates of nisin standards at each of the eight different concentrations (as indicated in Section 2.2) were also run in the 11 food sample matrices and the data used to develop calibration curves. Good linearity, as evident from R^2 values, was achieved for all calibration curves in the food samples. Table 1 summarises equations of the calibration curves with their corresponding R^2 values, limit of detections (LOD) and quantitations (LOQ) obtained for each sample. LODs ($S/N = 3$) range from 0.3 mg L^{-1} to 0.8 mg L^{-1} and LOQs ($S/N = 10$) range from 0.9 mg L^{-1} to 2.8 mg L^{-1}. Aqueous-like samples including pre-drop beer, red wine and Raspberry vinaigrette yielded the lowest LOD of 0.3 mg L^{-1}. The more complex samples, whipping cream, thousand islands, and crushed tomatoes, gave the highest LOD of 0.7–0.8 mg L^{-1}.

The reproducibility of the MEKC analysis was established by analysing three replicates of each food sample spiked with a known concentration of nisin. The coefficient of variation (CV) for the migration times and the peak areas were calculated and the results are listed in Table 1. It can be seen that the CV for the migration times was <1% for all the food samples and the CV for the peak area was <5%. The low %CV values indicate that this MEKC method is highly reproducible.

Quantitative recovery studies were carried out by spiking each food sample with a known amount (30 mg L^{-1}) of nisin standard. The samples were run and the peak areas were determined. The peak areas obtained for nisin and the equations of the calibrations curves developed in each sample were used to calculate the estimated quantities found which were then used to calculate the percent recovery values. As seen in Table 2, high recoveries were obtained for homogenised milk, raspberry vinaigrette salad dressing, plain yogurt and pre-drop beer perhaps due to less interfering analytes in these matrices. Samples that are more complex in appearance such as whipping cream, thousand islands and canned tomatoes yielded lower percent recoveries. Although the 2% milk, yogurt drink, and red wine were conceivably aqueous-like in nature, they still yielded lower recoveries.

3.3. Analysis of spiked food samples

Several food matrices were spiked with known nisin concentrations and analysed using the optimum buffer conditions. Extraction procedures involving chemical reagents were not necessary; all food samples were purified using specific molecular-weight centrifugal units depending on the viscosity and complexity of

Table 1
Validation parameters for proposed MEKC method in various food matrices.

Matrix	Nisin migration time (min)	Regression equation $y = mx + b$[a]	R^2	Migration time CV (%)	Peak area CV (%)	LOD (mg L^{-1})	LOQ (mg L^{-1})
Phosphate buffer	5.4	$y = (183.6 \pm 5.4)x - (33 \pm 172)$	0.9930	0.05	3.22	0.1	0.3
Milk samples							
Whipping Cream	5.4	$y = (106.4 \pm 3.7)x + (363 \pm 118)$	0.9951	0.38	0.17	0.8	2.8
Two percentage milk	5.5	$y = (143.2 \pm 6.1)x - (817 \pm 204)$	0.9827	0.46	1.44	0.4	1.4
Homogenised milk	5.6	$y = (154.1 \pm 4.1)x - (1384 \pm 136)$	0.9927	0.05	0.74	0.4	1.3
Salad dressings							
Raspberry vinaigrette	5.4	$y = (104.9 \pm 2.2)x - (274 \pm 68)$	0.9961	0.32	3.37	0.3	1.1
Thousand islands	5.3	$y = (67.4 \pm 3.0)x - (28 \pm 91)$	0.9765	0.56	0.91	0.7	2.4
Yogurt							
Plain yogurt	5.5	$y = (80.5 \pm 3.6)x - (241 \pm 117)$	0.9983	0.29	1.98	0.6	2.0
Yogurt drink	5.6	$y = (114.7 \pm 4.1)x - (495 \pm 126)$	0.9911	0.63	4.41	0.4	1.4
Alcoholic beverages							
Pre-drop beer	5.5	$y = (151.4 \pm 2.8)x - (154 \pm 86)$	0.9989	0.38	0.40	0.3	1.0
Red wine	5.4	$y = (76.4 \pm 2.5)x - (66 \pm 76)$	0.9852	0.25	2.05	0.3	0.9
Others[b]							
Processed cheese	5.7	$y = (112 \pm 3.8)x + (404 \pm 116)$	0.9973	0.64	1.65	0.4	1.4
Canned tomatoes	5.6	$y = (62.8 \pm 2.8)x - (310 \pm 84)$	0.9899	0.13	2.27	0.8	2.6

[a] y and x stand for peak area and concentration (mg L^{-1}) of nisin, respectively.
[b] LOD and LOQ are in mg kg^{-1}.

Table 2
Comparison in percent recovery for food samples ($n = 3$).

Matrix	Quantity added (mg L^{-1})	Quantity found (mg L^{-1})	Recovery (%)
Milk samples			
Whipping Cream	30	30.9	103.0
Two percentage milk	30	27.5	91.6
Homogenised milk	30	29.5	98.5
Salad dressings			
Raspberry vinaigrette	30	29.7	98.9
Thousand islands	30	25.0	83.2
Yogurt			
Plain yogurt	30	29.4	97.9
Yogurt drink	30	27.0	89.9
Alcoholic beverages			
Pre-drop beer	30	29.7	99.1
Red wine	30	27.9	92.9
Others			
Processed cheese	30	31.2	104.0
Canned tomatoes	30	28.1	93.8

the matrix. Filtration time varied among the samples. The minimum filtration time was approximately 10 min for less viscous samples such as the red wine and pre-drop beer. The samples were run using the optimised MEKC conditions of 50 mM sodium phosphate, 80 mM SDS, and at pH 3.75. A typical electropherogram for a food sample (i.e., whipping cream) spiked with 50 mg L^{-1} nisin is shown in Fig. 3B with nisin detected around 5.5 min.

4. Conclusions

Successful identification and quantitation of nisin in an uncoated capillary using MEKC has been achieved. The method developed did not involve any complicated extractions and nisin could be detected in 6 min. Optimised MEKC conditions were applied to several food matrices including dairy products (milk, processed cheese, and yogurt), alcoholic beverages, salad dressings and canned tomatoes. Limits of detection (LOD) and quantification (LOQ) were in the range of 0.3–0.8 and 1.0–2.8 mg L^{-1}, respectively. This study offers one of the most practical and rapid detection of nisin, at lower ppm level, which is comparable with other existing analytical techniques. This developed method can be used for quality control of nisin in the food and agricultural industries.

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