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Short communication

Rapid and sensitive determination of hydroxyproline in dairy products using micellar electrokinetic chromatography with laser-induced fluorescence detection

Ya-lei Dong[a], Na Yan[d], Xi Li[a], Xi-min Zhou[a], Lei Zhou[a], Hai-juan Zhang[a], Xing-guo Chen[a,b,c,*]

[a] National Key Laboratory of Applied Organic Chemistry, Lanzhou University, Lanzhou 730000, China
[b] Department of Chemistry, Lanzhou University, Lanzhou 730000, China
[c] Key Laboratory of Nonferrous Metal Chemistry and Resources Utilization of Gansu Province, Lanzhou 730000, China
[d] Gansu Entry-Exit Inspection and Quarantine Bureau, Lanzhou 730020, China

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ABSTRACT

Many reports have focused on the determination of hydroxyproline (Hyp) in blood plasma, urine sample, meat and meat products, however, there are few concerned with the Hyp assay in dairy products for food quality assurance up to now. In this paper, we described a sensitive and automated approach for the determination of Hyp in milk powder, liquid milk, milk drink and soymilk powder samples by micellar electrokinetic chromatography (MEKC) based on in-capillary derivatization for the first time. Under the optimal conditions, derivatization and separation procedure could be completed within 7 min and the detection limit for Hyp was 1.6 ± 0.5 ng mL^{-1}. Comparing with the existing alternatives, the present method exhibited some relevant advantages, including full automation, satisfactory sensitivity, and short analysis time for Hyp assay in dairy products.

1. Introduction

Hydroxyproline (Hyp), which is formed by hydroxylation of proline, is found almost exclusively in collagen and provides an important marker to directly measure collagen content [1,2]. Recently, it was reported that some illegal factories found a way to extract protein from leather scraps, and then added the hydrolyzed leather protein into dairy products, whereby boost the nitrogen content for increasing their protein amount [3]. Unfortunately, people who drink this dairy product will run the risk of developing osteoporosis. In consideration of the public health, the Chinese Ministry of Health promulgated a regulation to determine leather hydrolyzed protein in milk and dairy products by spectrophotometry [4]. It is based on the fact that leather is mainly consisted of collagen which is not present in pure milk. However, the spectrophotometric method [5] has poor sensitivity and requires more complicated operation, large sample amount, and time-consuming preliminary clean-up step, which is not conductive to detect large number of samples. Therefore, it is necessary to establish an automated, fast and sensitive method to particularly determine Hyp in dairy products.

Many chromatographic methods have been developed to detect Hyp in different samples up to now, such as gas chromatography (GC) [6], anion-exchange chromatography (AEC) [7–9], high-performance liquid chromatography (HPLC) [10–14], capillary electrophoresis (CE) with electrochemiluminescence detector [15–17], MEKC with two fluorescent labeling regents [18,19], and so on. Unfortunately, these methods all focused on samples of urine, skin tissue, muscle, protein hydrolysate, serum, or exhaled breath condensate samples. No attempts have been made to determine Hyp in dairy products up to now.

In recent years, in-capillary derivatization technique coupled with CE–LIF detection has received attractive attention because of some remarkable advantages over conventional pre- or post-capillary techniques [20]. Many works [21,22] demonstrated that it was an extremely useful technique for analytical chemistry. Furthermore, 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) is extensively studied as a popular labeling reagent due to its excellent advantages [11], including good reactivity with both primary and secondary amines, high reaction rate and few fluorescent side products.

So far, to the best of our knowledge, no study used NBD-F for the in-capillary derivatization of Hyp. Moreover, few reports concerned with the Hyp analysis in dairy products for food quality assurance

* Corresponding author at: Lanzhou University, Deparment of Chemistry, South Tianshui Road #222, Lanzhou, Gansu 730000, China.. Tel.: +86 931 891 2763; fax: +86 931 891 2582.
E-mail address: chenxg@lzu.edu.cn (X.-g. Chen).

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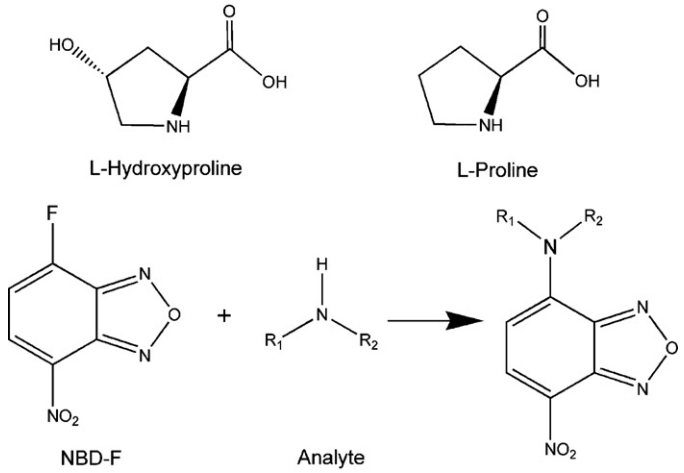

Fig. 1. Chemical structures of Hyp and Pro, and the scheme of the derivatization reaction with NBD-F.

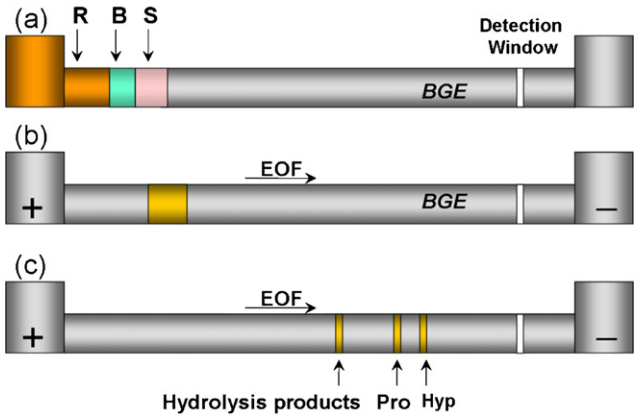

Fig. 2. Schematic illustration for in-capillary derivatization of Hyp and Pro with NBD-F. (a) Hydrodynamic injection of the sample (S), derivatization buffer (B) and NBD-F (R) plugs using S–B–R mode; (b) mixing and reaction of analytes with NBD-F after applying the running voltage; (c) separation and detection of the derivatives.

purpose. In this paper, we developed a new MEKC–LIF method for sensitive determination of Hyp, utilizing in-capillary derivatization with NBD-F as fluorescent reagent. Furthermore, this method was applied for the determination of Hyp in milk powder, liquid milk, milk drink and soymilk powder.

2. Experimental

2.1. Apparatus and materials

All experiments were performed on a Beckman P/ACE MDQ system (Fullerton, CA) equipped with a LIF detector (λ_{ex} = 473 nm, λ_{em} = 520 nm). The excitation light was from a laser (MBL-III-473, 20 mW, Chuangchun New Industries Optoelectroniecs Tech. Co., Ltd, Changchun, China). Data acquisition and instrument control were carried out using 32 Karat software (Version 7.0). An uncoated fused-silica capillary with dimensions of 50.2 cm length (effective length 40.0 cm) × 50 μm I.D. was purchased from Sino Sumtech Optical Conductive Fiber (Handan, China), and was thermostated at 25 °C. A PHS-3C acidity meter (Shanghai REX Instrument Factory, Shanghai, China) was used for the pH measurement.

All solvents and reagents were of analytical grade. L-Proline (Pro) and L-hydroxyproline (Hyp) were obtained from Sinopharm Chemical Reagent Co., Ltd. and Sigma, respectively, and the chemical structures are shown in Fig. 1. NBD-F was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Deionized water was used throughout.

2.2. Solutions preparation

Pro and Hyp with the concentration of 200 μg mL^{-1} were prepared and stored at 4 °C. 10 mM NBD-F stock solution was prepared in acetonitrile (ACN), stored at −18 °C, covered with aluminum foil, and diluted to the desired concentration with ACN before use.

2.3. Sample preparation

Milk powder, liquid milk, milk drink and soymilk powder were purchased from local market. An aliquot of samples, corresponding to 0.1 g of milk powder and soymilk powder, 0.1 mL of liquid milk and 2.0 mL of milk drink, added with varying amount of standard Hyp, were hydrolyzed referring to GB/T 5009.124-2003 of China. The hydrolysate solutions were diluted 10,000-fold before CE analysis.

2.4. Capillary electrophoresis conditions

Prior to its first use, the new capillary was conditioned by rinsing sequentially with methanol for 5 min, water for 3 min, 0.1 M HCl for 20 min, water for 5 min, 0.1 M NaOH for 20 min, water for 5 min, and running buffer for 20 min at 138 kPa, finally, equilibrated at 20 kV with running buffer for 20 min. To assure a good repeatability, the capillary was rinsed at the beginning of each experimental session and between two runs according to the previous reported conditions [23].

The in-capillary derivatization with zone-passing mode was performed using the following procedure [22]. Briefly, under hydrodynamic conditions, sample solution (S, 5 s at 3.45 kPa), derivatization buffer (B, 3 s at 2.07 kPa) and NBD-F solution (R, 3 s at 2.07 kPa) were successively introduced to the anodic end of the capillary (this mode was named as S–B–R mode). Subsequently, the mixed region was immediately separated by applying a running voltage of 20 kV. The mechanism of in-capillary derivatization method is illustrated in Fig. 2.

3. Results and discussion

3.1. Strategies for in-capillary derivatization

To select the optimum in-capillary derivatization strategy for Hyp with NBD-F, mixed mode and zone-passing mode were examined respectively. The mixed mode [23,24] attempted to electrokinetically mix the sample and reagent zones under a lower potential and then wait for several minutes for complete reaction before applying the running voltage. In contrast, zone-passing mode is performed by applying the separation potential just after the introduction of the sample, buffer and reagent plugs. This mode is appropriate for fast kinetic processes where high reaction yields are achieved in a few seconds [22]. The comparison of the results showed that the fluorescence intensity of Hyp derivatives had no obvious variation over the two modes. Therefore, zone-passing mode was adopted as the optimum strategy.

Subsequently, various introduction sequences for sample, derivatization buffer and reagent were investigated. Results showed that the S–B–R configuration provided the maximum peak area for Hyp, suggesting the faster migration velocity of NBD-F than analytes. Hence, zone-passing mode with S–B–R introduction sequence was chosen as the optimum strategy for in-capillary derivatization, as shown in Fig. 2.

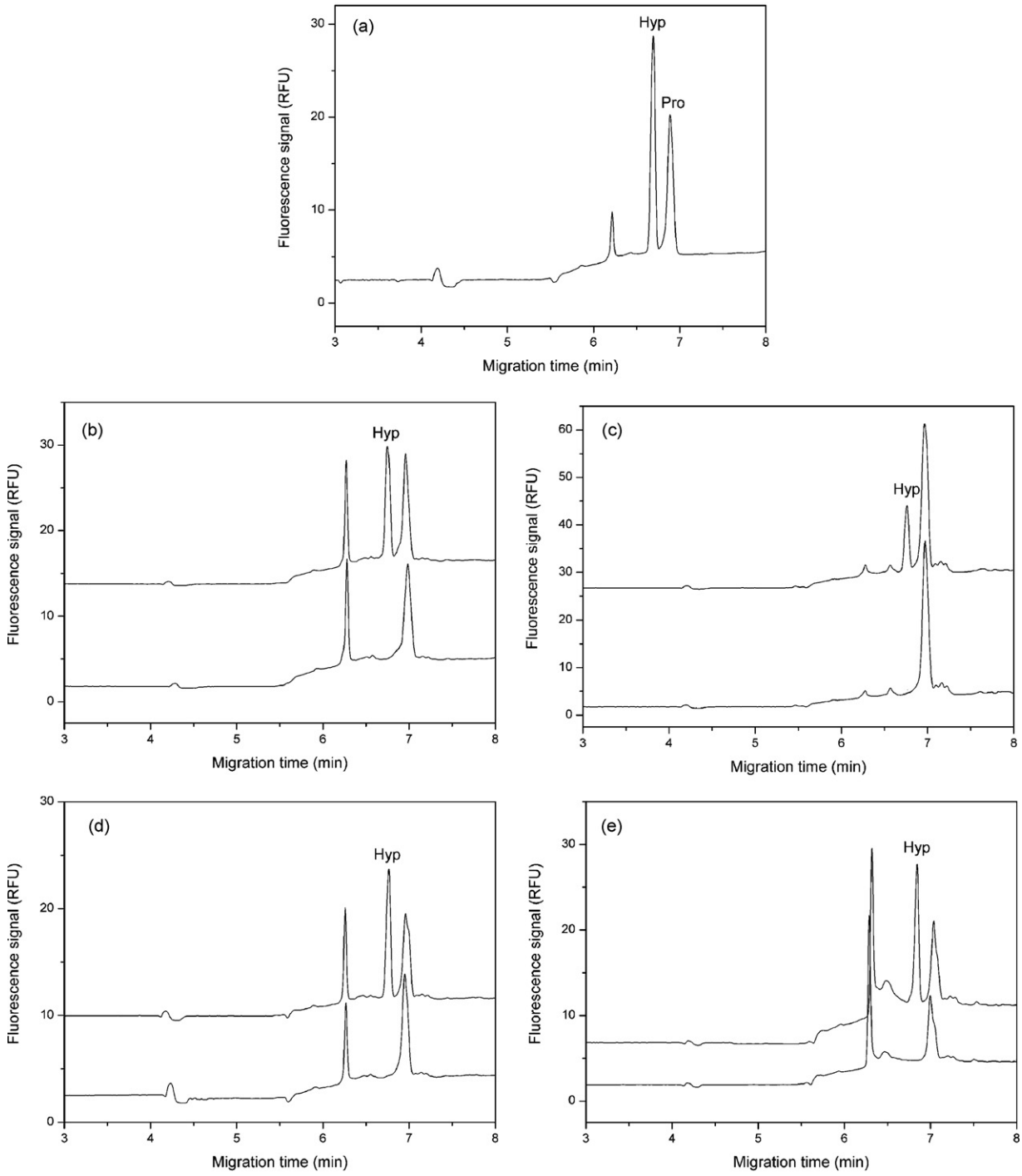

Fig. 3. Typical electropherograms of the standard, sample, and spiked sample under optimum experimental conditions: (a) standard of Hyp and Pro solution; (b) milk powder sample; (c) soymilk powder sample; (d) milk drink sample; (e) liquid milk sample. The upper lines in panels b–e correspond to practical samples spiked with 200 ng mL^{-1} of Hyp. Separation conditions: 25 mM sodium tetraborate, 30 mM SDS, at pH 9.7 and an applied voltage of 20 kV, capillary temperature: 25 ± 0.1 °C.

3.2. Method optimization

Taken into account the similar chemical structure (as shown in Fig. 1), proline was considered as the most important interfering material affecting the determination of hydroxyproline. Therefore, with respect to resolution, analysis time and repeatability, 25 mM sodium tetraborate of pH 9.7 containing 30 mM SDS was selected as the running buffer, and a solution of 20 mM sodium tetraborate at pH 9.7 was recommended as the optimal derivatization buffer. 5 mM of NBD-F was chosen to obtain the optimal fluorescence intensity. Under the optimum conditions, as shown in Fig. 3a, the standard mixture could be separated within 7 min.

3.3. Method validation

The linearity between peak area and the concentration were found to have excellent linear relationships over the range of 5–2000 ng mL^{-1} with correlation coefficient 0.9986–0.9999 in different matrix. The limit of detection (LOD), were 1.6 ± 0.5 ng mL^{-1}. The sensitivity of the present method was higher than those

Table 1
Results for the determination of the Hyp in original samples.

Sample	Initial amount ($\mu g\,mL^{-1}$)	Added ($\mu g\,mL^{-1}$)	Found ($\mu g\,mL^{-1}$)	Recovery (%)	RSD[a] (%)	Average recovery (%)
Milk powder	–[b]	0.05	0.047	94	5.02	95
		0.1	0.086	86	4.32	
		1	1.064	106	3.71	
Soymilk powder	–	0.05	0.056	112	1.94	103
		0.1	0.091	91	2.04	
		1	1.049	105	1.70	
Milk drink	–	0.05	0.049	98	7.25	100
		0.1	0.100	100	3.44	
		1	1.012	101	0.62	
Liquid milk	–	0.05	0.055	110	3.31	97
		0.1	0.088	88	1.85	
		1	0.926	93	4.15	

[a] The relative standard derivation was calculated from three recovery data.
[b] No detectable.

determined by the spectrophotometry and other methods. The repeatability of the method was determined with a spiked sample solution at the levels of 5, 500 and 2000 $ng\,mL^{-1}$. The relative standard deviation (RSD) of migration time and peak area, were $0.83 \pm 0.56\%$, $3.29 \pm 1.30\%$ (intraday) and $1.87 \pm 1.04\%$, lower than 4.85% (interday), respectively. In addition, the method produced narrow peak for Hyp, which was observed with a high number of plates of 68834, under the optimal experimental conditions.

3.4. Applications and recoveries

Milk powder, liquid milk and milk drink were employed for assessing the applicability of the proposed method. Soymilk powder was also selected because it was one of the traditional diets for the Chinese.

The typical electropherograms are shown in Fig. 3. The peaks were identified by comparing migration times and also by spiking the standards to sample solutions. No detectable amount of Hyp was found in these samples. The recoveries of the method were determined with standard addition method for Hyp spiking in original samples at three levels, and three replicates were performed at each level. As shown in Table 1, the average recoveries of Hyp ranged from 95% to 103%, suggesting that Hyp in dairy products and soymilk powder could be reliably detected.

It also confirmed that other coexisting amino acids in protein hydrolysate did not influence the determination of Hyp. We select three kinds of amino acids as interfering materials, including aliphatic amino acids (representative amino acids such as leucine, cysteine and glutamic acid), aromatic amino acids (phenlanine and tyrosine) and heterocyclic amino acid (histidine). The mixed solution with interferential amino acids exhibited the result same as the solution consisted of Hyp and Pro, implying that other amino acids did not affecting the determination of Hyp.

3.5. Comparison with other methods

It will be significant to compare the analytical features of the present method with other reported chromatographic alternatives (Table 2). It showed that LIF detector provided comparable or higher sensitivity comparing with other types of detector. However, HPLC employed NBD-F or other reagents required pre-column derivatization procedures, which were more laborious. In addition, the previous MEKC methods employed two steps in labeling procedure, involving initial reaction with OPA to remove primary amino acids, and then the secondary amine was reacted with PITC or NBD-Cl. Comparing with the in-capillary derivatization method developed in Ref. [22], our approach was sensitive and greatly shortened the analysis time. When large quantities of sample are present, the present method is preferred and can save lots of time and labor. In a word, our method exhibits some relevant advantages such as full automation, satisfactory sensitivity, and short analysis time for

Table 2
Summary table of the existing chromatographic methods for Hyp determination.

Term	Analytical method	Derivatization reagent or detector	Matrix	LOD	Retention time of Hyp/Pro	Ref.
Pre-column derivatization	HPLC	NBD-F	Skin tissue	–[a]	7/30 min	[10]
	HPLC	NBD-F	Protein hydrolysate	10 fmol	5/20 min	[11]
	HPLC	NBD-F	–	5 fmol	9/28 min	[12]
	LC	Phisyl-Cl and DPS-Cl	Serum and urine	30 fmol	10/18 min	[13]
	2D-HPLC	NBD-F	Serum and skin tissue	1 fmol	11/44 min	[25]
	IEC	NBD-Cl	Blood plasma	0.5 nmol	32/50 min	[9]
	MEKC	OPA and PITC	Skeletal muscle	1 pmol	10.5/11 min	[18]
	MEKC	OPA and NBD-Cl	Muscle	–	7/– min	[19]
In-capillary derivatization	CE	NQS	Animal feed sample	0.14 pmol	29/32 min	[22]
	MEKC	NBD-F	Dairy products	8.2 $nmol\,L^{-1}$	6.7/6.9 min	Present work
Other detector	HPAEC	PAD	Meat-based food	–	8.5/10 min	[7]
	HPAEC	PAD	Plant cell wall	–	7/– min	[8]
	HILIC	MS/MS	Exhaled breath condensate	38.1 $\mu mol\,L^{-1}$	8/7.5 min	[14]
	GC	FPD	Urine samples	0.2 pmol	10/7 min	[6]
	CE	ECL	–	2 $\mu mol\,L^{-1}$	17/16 min	[15]
	CE	ECL	Urine samples	4 $\mu mol\,L^{-1}$	8.3/7.7 min	[16]
	CE	ECL	–	μmol	7/6 min	[26]
	CE	CCD	Amniotic fluid	8.6 $\mu mol\,L^{-1}$	67/49 min	[17]

[a] No data presented.

Hyp assay. These features make it suitable for quality control of dairy products.

4. Conclusions

In the present work, a MEKC–LIF method using in-capillary derivatization with NBD-F as fluorescent reagent has been developed for the analysis of Hyp in milk powder, liquid milk, milk drink and soymilk powder. By comparison with the previous method, the present method exhibits distinct advantages including lower limit of detection, short analysis time, automatic operation and good repeatability for Hyp assay. These remarkable features make it suitable for quality control in the manufacturing of dairy products.

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