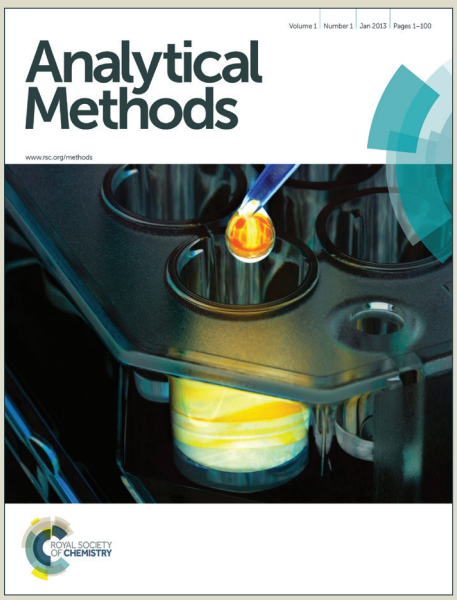
Analytical Methods

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Anlytical Methods

RSCPublishing

PAPER

Fluorometric determination of proline in honey by high-performance liquid chromatography after pre-column derivatization with7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F)

YiLi[ab],JinhuiZhou[b],XiaofengXue[b],LimingWu[b],LanzhenChen[c],JinzhenZhang*[b] , ShumingYang*[a]

An optimization method of NBD-F derivatizationhas been developed for the determination of proline in honey using high performance liquid chromatography (HPLC) with fluorescence detection.To determine the free proline in different samples of honey, ultrasound extraction was performed using 0.1 M borate buffer solution as extraction solvent. The chromatographic separation was performed on a XDB C18 column (150 × 4.6 mm i.d.) using 0.1 M sodium acetate buffer solution (pH 7.2):methanol:tetrahydrofuran (900:95:5 respectively, v/v/v) as mobile phase A and methanol as mobile phase B at a flow rate of 1.0 mL/min with a run time of 10 min. The assay was linear over a range of 0.15 μg/mL to 100.00 μg/mL with a lower limit of detection of 3.00 mg/kg, and recoveries were greater than 90%. The intra-day and inter-day precision was less than 4%. This method was successful in determining free proline in honey, ranging from 80.5 mg/kg to 426.4 mg/kg indifferent honey samples.

1. Introduction

Honey contains numerous active components with at least 181 substances, such as sugars, proteins, amino acids, minerals and vitamins.[1] Free amino acids are not only important nutritional components of honey, but are also indicators of the quality and floral honeyorigins. Indeed a number of studies have shown a correlation between amino acid composition and the botanical or geographical differences of different honey samples.[2-6] Iglesias *et al* reported that amino acids in honey account for 1% (w/w),[7] with proline as the major amino acid.[6] Proline is an important amino acid that contributes to the antioxidant properties of honey.[8] Moreover, proline content has been used as the indicator of honey ripeness and sugar adulteration when it falls below a value of 180 mg/kg.[9-10] Therefore, proline content is a critical marker for the authentication of honey quality.[11-13]

A series of methods based on spectrophotometric and chromatographic assays have been described to analyze proline content in biological matrices.[14-15] The chemical structure of proline lacks native fluorescence and significant UV absorption. Consequently the derivatization procedure to increase the signal response of target analytes by introducing chromophores or fluorophores was used for the spectrophotometric and chromatographic detection of proline. In the past, the spectrophotometric detection method using ninhydrin as the derivatization reagent (AOAC,1984) was adjusted for proline determination in honey.[16] Recently, high performance liquid

chromatography (HPLC) has become increasingly popular in the analysis of proline in honey due to its spectrophotometric specificity, detection sensitivity and automatic operation for qualitative analysis.[17-19] The major classes of UV and fluorescent derivatization reagents for the determination of proline in honey samples by HPLC include diethylethoxymethylenemalonate (DEMM) [5], fluorenylmethyl-chloroformate (FMOC-Cl)[18-19]and o-phthaldialdehyde with N-acetyl-l-cysteine.[20] The reaction condition for the DEMM method is 90°C for 50 min. The most convenient method appears to be the FMOC-Cl method, which takes only 2 minutes at room temperature.[21] However FMOC-CI is an alcoholic derivatization, it was reported to be have detrimental effects on the chromatographic column. [21]

A relatively new fluorescent derivatization, 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) is highly reactive with primary and secondary amines under mild reaction conditions, forming stable fluorescent NBD-products with excellent sensitivity. In addition, when the reagent is hydrolyzen, its fluorescence can be erased under acidic condition.The derivatization scheme for reaction of NBD-F with amino acids is shown in Fig1. For this reason, NBD-F has become a widely used derivatization reagent for amines and amino acid measurements in many biological matrices [22-25].The limit of detection (LOD) and limit of quantification (LOQ) of proline have been reported as low as 5 fmol and 16 fmol, respectively.[26] This fluorescent labelling reagent has not yet been used to detect proline in honey by HPLC.

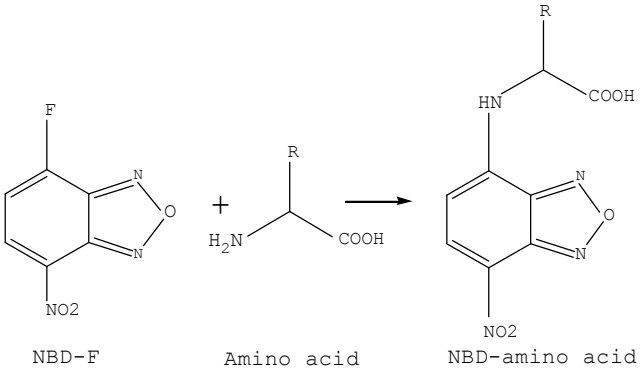


Fig.1 The derivatization scheme for reaction of NBD-F with amino acids

In this study, a rapid and sensitive HPLC method with NBD-F as the fluorescent derivatization agent was developed to analyse proline content in honey. In addition, a simple and environment friendly pre-extraction method was employed with merits of short extraction time and free use of organic reagents, and the proline contents for six different origin of raw honeys were analysed with this method.

2. Experimental

2.1 Chemicals and reagents

The L-proline (Pro) was purchased from Aladdin (Purity: 99.0%, Aladdin Reagent Database Inc.) and NBD-F (Purity≥98.0%) were purchased from Sigma-Aldrich ((St. Louis, MO,USA). Methanol and acetonitrile were purchased from Merck (Darmstadt, Germany) and HPLC grade tetrahydrofuran was purchased from Fisher Scientific(Waltham, MA, USA). Ultrapure water was purified using a Milli-Q system (Millipore, MA, USA) and all other analytical grade chemicals were purchased from Sinopharm Chemical Reagent, Beijing Co., Ltd, China. Aqueous solutions were prepared using ultrapure water. The 0.1 M borate buffer solution containing 0.001 M disodium ethylenediaminetetraacetic acid (EDTA) was adjusted to pH8.0 with 0.1 M sodium hydroxide.

2.2 Equipment and Chromatographic Conditions

A homogenizer (Vortex-5, HaimenQilinbeier Instruments Co., Ltd, China), ultrasonic bath (KQ218, Kunshan Ultrasonic Instruments Co., Ltd, China), and re-circulating water bath (HH-W, Jintan City Kaiyuan Experiment Instrument Factory, China) were used for the extraction and derivatization of proline in honey.

A HPLC system (Agilent 1200, USA) equipped with a G1321A fluorescence detector, Eclipse XDB C18 (150 mm×4.6 mm, 5μm) and C18 (250 mm×4.6 mm, 5μm) columns were used for chromatographic analysis. The gradient program using 0.1 M sodium acetate buffer solution (pH adjusted to 7.2 with acetic acid):methanol:tetrahydrofuran (900:95:5 respectively, v/v/v) as mobile phase A and methanol as mobile phase B was employed as follows: 0~0.5 min, phase A was decreased from 100% to 75% and maintained for 7 min at 75%; 7~8 min, phase A was decreased to 0% until 10 min was reached;10~12 min, phase A was increased to 100% and maintained at 100 % until 15 min was reached. The flow rate was set at 1.0 mL/min and the column temperature was maintained at 30°C. The running time per assay was 15 min. The injection volume for samples and standards was 20 μL. The fluorescence detector was operated with an excitation wavelength of 470 nm and an emission wavelength of 530 nm. For quantification,

the peak area was analyzed by the software Chemstation (Agilent, USA).

2.3 Honey samples

All honey samples were directly supplied by a beekeeper from China in 2012, and stored at room temperature until analyzed. Microscopic analysis confirmed the pollen origins, which included six lime tree honey, six oil seed rape honey, four lychee honey, five chaste tree twig honey, four Chinese date honey and three wild flower honey derived from different floral species. The moisture content was determined at 40±1°C using an Abbe refractometer (Shanghai Precision Scientific Instrument Co., Ltd.) according to the standard method from the China Entry-Exit Inspection and Quarantine Bureau.[27]

2.4 Preparation of standard solutions

Proline (100 mg) was put into 100 mL volumetric flask and dissolved in 30 mL 0.1 M borate buffer solution, and diluted with the same solvent to obtain stock solutions with a concentration of 1.0 mg/mL. The calibration curve was obtained by diluting the proline stock solutions with 0.1 M borate buffer solution to produce the following concentrations:100.0 μg/mL, 40.0 μg/mL, 10.0 μg/mL, 5.00 μg/mL, 2.50 μg/mL, 0.625 μg/mL and 0.15 μg/mL. Stock solutions were stored at 4°C and were not used after 3 months. Working solutions were freshly prepared before use.

2.5 Sample preparation

For proline extraction, honey samples (1.0 g) were dissolved in 20 mL borate buffer solution and treated with ultrasound for approximately 10 min. Sample solutions were transferred into a 50 mL brown volumetric flask and further diluted to 50 mL with borate buffer solution. The extraction solution was then filtered through a 0.2 μm nylon filter membrane.

2.6 Derivatization method

The derivatization reagent, NBD-F, was selected as previously reported with slight modifications for derivatization conditions.[28]Briefly, 200 μL of each calibration solution or extracted sample solution, 70 μL acetonitrile and 30 μL NBD-F solution (100 mM in acetonitrile, stored at -20°C, restored to room temperature before use) were consecutively added into a brown glass vial. The mixture was allowed to react for 12 min at 60°C and then placed on ice, followed by termination of the derivatization reaction by adjusting the medium to approximately pH 1.0 with 0.1 M ice-cold HCl (100 μL). An aliquot of 20 μL of the derivatized sample was injected into the HPLC instrument for analysis.

2.7 Method Validation

For the validation of linearity, calibration solutions ranging from 0.15 μg/mL to 100.0 μg/mL were analyzed in five replicates. The calibration curves were constructed from peak areas of standards relative to their concentrations. Sensitivity of the method was determined by the limit of detection (LOD) at a signal-to-noise ratio of 3 and the limit of quantification (LOQ) at a signal-to-noise ratio of 10 by diluting honey matrix. LOD and LOQ levels are absolute amount of proline in honey sample

Precision was assessed by repeatability and reproducibility of the intra-day and inter-day variability. Repeatability and reproducibility were evaluated on 3 different honey samples, and the reproducibility was determined for 3 independent days.

To evaluate the accuracy of the method, recovery tests were performed by adding three concentrations (125.0 mg/kg, 250.0 mg/kg and 500.0 mg/kg) of standard proline solution to a known proline content of honey sample. Each concentration was repeated

Stability analyses of proline derivatives of the standard solution and the sample solution stored at room temperature were performed.the solution was stored over a period of 24 h at room tem-perature and then analyzed.

Peak purity of proline derivatives was assessed to evaluate the specificity of the method. The sample and standard chromatograms were scanned at peak start , peak apex and peak end positions. The specificity of the method was determined by comparison of the chromatogram of standard and sample solution.

The robustness of the developed method was assessed by introducing very small changes in the analytical methodology at some honey samples spiked with 125 mg/kg of proline (3 replicates).

The alteration of the retention time, peak area and tailing peak at slight variation of the mobile phase composition pH (7.1,7.2,7.3), column temperature(29 °C,30 °C,31 °C) and flow-rate（0.9 mL/min,1.0 mL/min,1.1 mL/min）were determined. The percentage

of relative standard deviation(%RSD) of the experiment was calculated to assess the robustness of the method.

2.8 Statistical analysis

Data were analyzed using the Microsoft excel data analysis package and are presented as mean ± standard deviation (SD).

3. Results and discussion

3.1 Optimization of chromatographic conditions

High signal intensity of the NBD-proline derivative has previously been detected with a fluorescence detector at an excitation wavelength of 470 nm and an emission wavelength of 530 nm.[22-23] To optimize the chromatographic conditions, different mobile phases and chromatography columns were evaluated and compared to determine the best peak resolution. Initially, on the basis of the method previously described by Imaie and Watanabl,[22] the chromatographic separation was performed on a C18 (250 mm×4.6 mm, 5μm) column using 0.1 M phosphate buffer (pH6.0) as mobile phase A and tetrahydrofuran:methanol (50:50,v/v) as mobile phase B at a flow rate of 0.8 mL/min with a gradient elution program. The symmetry factor calculated by the liquid chromatography software was only 0.75. To improve the symmetry factor, 0.1 M sodium acetate buffer (pH 7.2): methanol: tetrahydrofuran (900: 95: 5 by volume) was used for mobile phase A and methanol as the mobile phase B for gradient elution, were used on a XDB C18 (150 mm × 4.6 mm, 5 μm) column at a flow rate of 1.0 mL/min according to the method described by Vázquez-Ortíz *et al*. [27] The symmetry factor increased from 0.75 to 0.90. Proline in the honey samples was identified by comparing retention times against those obtained from proline standard solutions. Typical chromatograms of standard and sample under the optimized chromatographic conditions are shown in **Fig.2**.

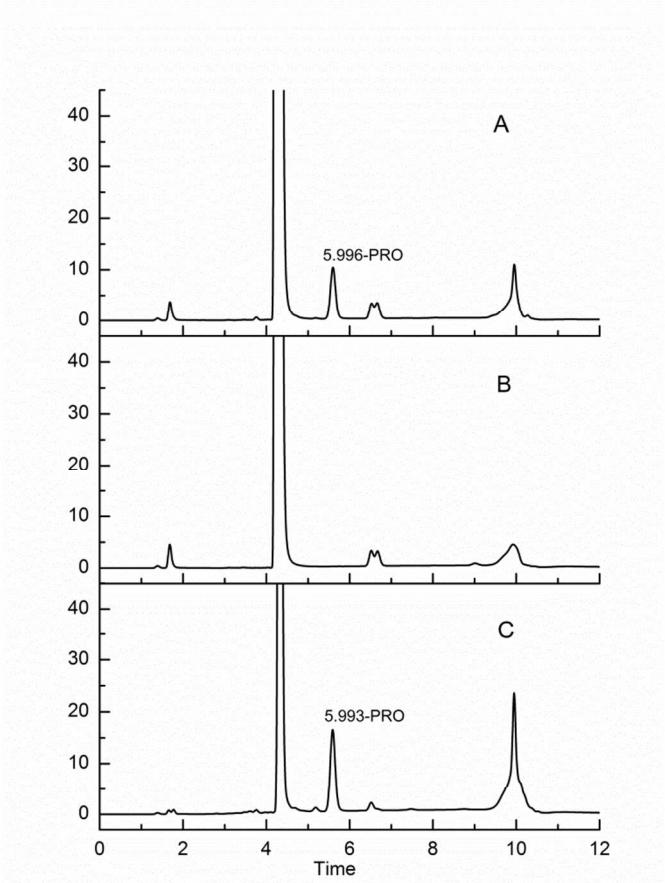


Fig.2 Chromatograms of the proline derivatives.(A, standard solution of 5.0 μg/mLproline (B, 0.1 M borate buffer solution) and (C, honey sample solution)

3.2 Extraction of free proline

Honey samples and proline are soluble in water, and the use of borate buffer decreases the hydrolysis of NBD-F to NBD-OH in the derivatization reaction. To keep consistency, 0.1 M borate buffer (pH 8.0) was chosen for the extraction of proline. Recovery experiments with and without ultrasound-assisted extraction were compared. The results showed that recovery with ultrasound-assisted extraction for 10 min and 15 min was 82.4% and 81.6%, respectively, both of which were higher than that without ultrasound extraction (60.0%). Consequently, borate buffer combined with ultrasound-assisted extraction for 10 min was used for free proline extraction from honey.

3.3 Optimization of derivatization

The derivatization conditions for amantadine in honey have been previously reported by our group[28]. Initially, the proline in the

standard solution and the honey samples was derivatized in the same manner. Ultrasound-assisted extraction without purification may induce some impurities which would interfere with the quantitative analysis of proline in honey. Consequently, the effects of different weights of honey samples and different volumes of NBD-F on the analysing results were compared respectively.

The correlation between the peak area of proline derivatives and the weight of the honey sample was studied. As shown in **Fig.3**, in the weight range from 0.50 g to 1.0 g, there was no difference between peak area of proline derivatives per gram sample and the weight of the honey sample. However, at the weight range from 1.0 g to 6.0 g, a negative correlation between peak area of proline derivatives per gram sample and the weight of the honey sample was observed. A possible explanation for these phenomena is that some substances other than proline may react with NBD-F when the weight of the sample is increased. Subsequently, NBD-F could not completely react with the proline in heavier samples, resulting in a decrease in peak area of proline derivatives per gram sample. To ensure a complete reaction between proline and NBD-F, the weight of the samples should be controlled in the range from 0.5 g to 1.0 g.

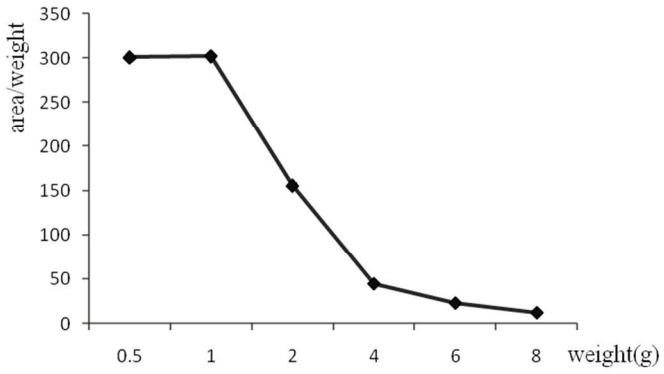


Fig.3 The correlation of different sample weight and peak area

Under the optimum sample extraction and weight conditions, the derivatization volume of standard or sample solution was decreased from 400 μL to 200μL. The effect of the derivatization reagent (100 mM) at different volumes (20 μL, 30 μL and 40 μL) was compared by measuring the peak area of proline derivatives. The results showed there was no obvious correlation between the peak area of proline derivatives of standard solution and volumes of derivatization reagent. But for the honey samples, peak area increased as the volume of the derivative agent increased (**Fig 4**). The peak area of proline derivatives adding 30 μL derivatization reagent was significantly higher than that of 20 μL derivatization reagent, while there was insignificant difference between 30 μL and 40 μL. It was decided that a volume of 30 μL of derivatization agent be used for the remaining experiments.

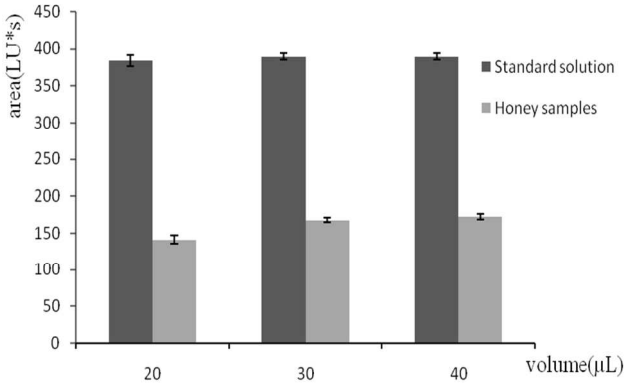


Fig.4 Effect of derivatization reagent volume on peak areas of proline standards and honey samples

3.4 Method Validation

To investigate the linear calibration range, proline solutions in the concentration range of 0.15 μg/mL to 100.0 μg/mL were prepared and analyzed using the optimized derivatization procedure and chromatographic conditions. The linear regression equation was y=19.287x-9.1325 and the regression coefficient of calibration curves was more than 0.999, indicating linearity in the concentration range from 0.15 to 100.0 μg/mL.The LOD and LOQ of proline were 3.00 mg/kg and 10.00 mg/kg, respectively.

The relative standard deviations (RSDs) of repeatability and reproducibility were less than 4% (Table 1). The recovery was within the range of 90.2% to 90.7%, with RSDs less than 4% (Table 2). These results indicate that the present method was valid for the quantification of proline content in honey.

Stability of proline derivatives of the standard solution and the sample solution stored at room temperature was analyzed by measuring the peak area at 0.25 h and 24 h, respectively. Consistent with a previous report,[28] there was no significant difference in the

peak area between 0.25 h and 24 h indicating that proline derivatives are stable when stored at room temperature for at least 24 h.

The specificity of the developed method for the analysis of proline in samples was confirmed by comparing the spectra obtainedin the standard and sample analyses (Fig.5).The peak start, peak apex, and peak end positions of these spectra were matching.

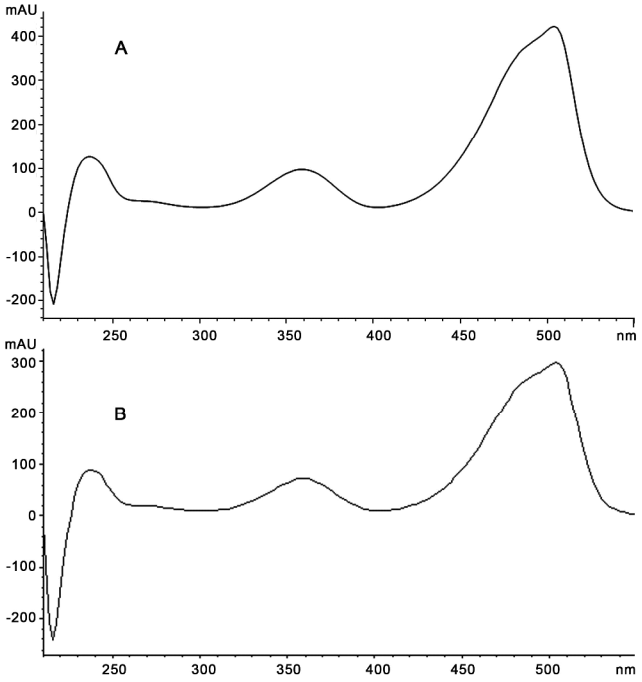


Fig.5 The emission spectra of NBD-Pro in standard(A)and sample(B)

The results obtained in robustness studies demonstrate that the chromatographic response of the analytes: retention time (R.S.D. <0.2%), sensitivity (peak area R.S.D. <1.4%) and asymmetry factor (R.S.D. <1.2%), were in all cases not strongly affected by slight variations in the composition of the mobile phase, the column temperature and the flow rate.

A Student's T-test assuming unequal variances was used to determine if statistically significant differences ($P < 0.05$) existed by comparing the proline contents of honey samples subjected to reference method (AOAC 979.20-1983) and this method in our previous work. According to the Student's t-test, there is no significant difference between the proline contents for both methods, when the calculated t is below the tabulated t value.

Table 1 Repeatability and reproducibility of proline determination in honey sample

Sample	Repeatability（n=3）		Reproducibility (n=9)	
	$\overline{X} \pm SD$ （mg/kg）	RSD（%）	$\overline{X} \pm SD$ （mg/kg）	RSD（%）
1	370.6±4.62	1.25	372.8±6.29	1.69
2	279.2±5.31	1.90	277.6±5.74	2.08
3	148.4±4.07	2.81	151.8±5.04	3.32

Table 2 Recovery of proline spiked honey (n=5)

Original（Mean ±SD，mg/ kg）	Spike level (mg/ kg)	Recovery (%)	RSD (100%)
370.6±4.62	500.0	90.5±0.45	2.09
	250.0	90.0±0.70	2.28
	125.0	90.3±0.23	3.10

Table3 Free proline content in different honey samples

Flora	Content of proline (mg/kg)			Numbers (＜180 mg/kg)	Moisture content （%）	Numbers (>21%)
	Minimum	Maximum	Mean			
Linden honey（n=6）	80.5	241.7	136.1±62.3	5	20.1~25.6	4
Oil seed rape honey（n=6）	37.3	149.6	88.3±52.9	6	21.8~25.6	6
Litchi honey（n=4）	137.3	206.6	164.7±30.5	3	22.9~23.7	4
Vitex honey（n=5）	180.9	269.8	235.1±36.4	0	19.8~22.2	2
Jujube blossom honey（n=4）	295.1	354.3	319.9±25.2	0	19.5~20.5	0
Multifloral honey（n=3）	256.8	426.4	322.7±90.9	0	18.3~19.4	0

3.5 Analysis of Real Samples

The validation method was used to analyze 28 raw honey samples collected in China in 2012. Average proline concentration in the analyzed honey samples ranged from 88.3 mg/kg in oil seed rape honey, to 322.7 mg/kg in multifloral honey (**Table 3**). The highest proline content was found in multifloral honey (426.4 mg/kg), and the lowest was in the oil seed rape honey (37.3 mg/kg). These results indicated that the multifloral honey samples contained the highest amount of proline, which is in agreement with the results reported in honey samples from Croatia.[30]The high amount of proline in multifloral honey samples may result from high amounts of pollen content in multifloral honeys. The minimum proline content in honey was determined by the EU in 2002, with 180 mg/kg considered the minimal value to be for international acceptance.[10] In the present study, proline levels in 50% of the raw honey samples were lower than the minimal international standard, including 5 samples of Linden honey, 6 samples of oil seed rape honey, and 3 samples of Litchi honey. This is in agreement with findings by Horn and Böhm who also reported that 47.3% of the measured honey samples (the majority of which were harvested from totally capped combs) had proline levels lower than 183 mg/kg and 55.1% of all samples had proline levels lower than 200 mg/kg. [31] For the samples of linden, oil seed rape and litchi honey, the average proline content was 136.1, 88.3 and 164.7 mg/kg, respectively. These proline levels were lower than that observed in vitex honey (235.1mg/kg), jujube blossom honey (319.9mg/kg) and multifloral honey (322.7mg/kg). In addition, the average moisture content in 14 samples of linden, oil seed rape and litchi honeys exceeded 21%(the maximum water content for honey)[32], and this was greater than the average moisture content of vitex, jujube blossom and multifloral honeys. These results indicate that there is a negative correlation between proline content and water content. It should be noted that some samples of honey from the same floral origin have higher moisture and proline content, and there is no correlation between proline and water content as described by Horn and Böhm.[31]

4. Conclusion

In this study, a simple and rapid HPLC method for the analysis of proline in honey was described. Pre-column derivatization with NBD-F was easy, fast and efficient, and the derivatives were stable. This procedure was applied to the analysis of honey samples with a simple extraction.

References

1 J.M. Alvarez-Suarez, S.Tulipani, S.Romandini, E.Bertoli and M. Battino,Mediterr.*J. Nutr. Metab*, 2010,**3**,15.
2 A. Pirini,L.S. Conte,O. Francioso and G. Lercker, *J. High Resol. Chromatogr*,1992, **15**,165.
3 A.Bouseta, V. Scheirman and S. Collin, *J. Food.Sci.*,1996,**61**, 683.
4 L .S. Conte, M .Miorini, A. Giomo, G. Bertacco and R. Zironi, *J. Agric. Food Chem.*,1998,**46**,1844.
5 I. Hermosín, R.M Chicón and M. Dolores Cabezudo, *FoodChem.*, 2003, **83**,263.
6 S.E.A.R. Mohammed and E.E, *J. Basic & Appl. Sci.*, 2010, **4,**552.
7 M.T. Iglesis, C.Delorenzo, M.C. Polo , P.J. Martin-Alvares, and E. Pueyo, *J. Food Agric. Chem.*, 2004,**52**,84-89.
8 M. Moniruzzaman,M.I. Khalil,S.A. Sulaiman and S.H. Gan, *BMC Complement Altern. Med.*,2013,**13**,43.
9 S.Bogdanov, C.LŰllmann, P.Marioleas, A.Tsigouri, J.Kerkvliet, A.Ortiz, T.Ivanov, P.Vit, P.Martin and W.von der Ohe, *Bee World*, 1999, **80**. 61
10 I. Hermosín,R.M. ChicónandM.D.Cabezudo,*Food Chem*.,2003,83,263.
11 L. Biino, Riv. *Ital. Essence Profumi.*,1971,**53**,80.
12 J.Chen, Y.B. Yang, Y.T. Hu, R. H.Xu, N.Cheng and W.Cao, *Apiculture of China*, 2010,61,11.
13 S. Bogdanov ,http://www.ihc-platform.net/ihcmethods2009.pdf, 2009.
14 E.Abrahám, C.Hourton-Cabassa,L.Erdei,andL.Szabados, *Methods Mol. Biol,* 2010,**639**,301.
15 A.Fabiani, A.Versari, G.P. Parpinello,M.Castellarand S. Galassi, *J. Chromatogr. Sci*, 2002,**40**,14.
16 AOAC 979.20-1983, Proline in Honey.
17 M.T. Iglesias, P.J.Martín-Alvarez, M. C. Polo, C.de Lorenzo, M.Gonzalezand E. Pueyo, *J.Agric.FoodChem.*,2006,**54**,9099.
18 S. Qamer,M. Ehsan,S. NadeemandA.R. Shakoori,*Pakistan J.Zool*,2007, **39**,99.
19 B.Carratù,M.Ciarrocchi,M.Mosca,E.Sanzini, *J. Api.Product Api.Medical Sci.*, 2011,**3**,81.
20 M.T. Kellya, A. Blaise, M. Larroque, *J. Chromatogr. A*, 2010, 1217, 7385.
21 J.L. Bernal, M.J. Nozal, J.C. Diego and A. Ruiz, *J.Sep.Sci*,2005,**28**,1039.
22 K. Imai and Y. Watanabe, *Anal.Chim. Acta.*, 1981, **130**,377.
23 K. Imai, J. Watanabe and T. *Toyo'oka, Chromatogr.*,1982,**16**,214.
24 Y. Song, T.FunatsuandM.Tsunoda, *Amino Acids*, 2012**,42**, 1897.
25 A.A.Elbashir, F.E.O.Suliman, H.Y.Aboul-Enein,*GU J. Sci.*, 2011,**24**,679.
26 Y. Song, T. Funatsu, M. Tsunoda. *J. Chromatogr. B*, 2011, **879**, 335.
27 The entry-exit inspection and Quarantine of the people's Republic of China,*Chinese Standard Press*, SN/T 0852-2000.
28 J.Z.Zhang, J.Zhao, J.H.Zhou, X.F.Xue. Y.Li, L.M. Wu and F.Chen.*Chin. J.Chem*,2011,**29**,1764.
29 F. A. Vázquez-Ortíz, O. E. Morón-Fuenmayorand N. F. González-Méndez, *J.Liq. Chrom. Relat. Tech.*,2005,**27**,2771.
30 A.M. González-Paramás, J.A.Gómez-Bárez, C. Cordón-Marcos, R.J. García-Villanova and J.S. José Sánchez, *Food Chem.*, 2006,**95**,148.
31 H.Horn,andD.Böhm,*DeutLebensem-Rundsch*, 2004,100,88.
32 O.E. Agbagwaand N. Frunk-peterside,*J.Microbiol.Biotech.Res*, 2011,**1**, 20.

[a]Institute of Quality Standards and Testing Technology for Agro-Products,Chinese Academy of Agricultural Science, Beijing China

[b]BeeResearch Institute, Chinese Academy of Agricultural Sciences, Beijing, China

[c]Laboratory of Risk Assessment for Bee Products, Ministry of Agriculture, Beijing, China

*Correponding to: JinzhenZhang Bee Research Institute, Chinese Academy of Agricultural Sciences, Beijing,100093, China.E-mail:jzzhang76@sina.com; and ShumingYang, Institute of Quality Standards and Testing Technology for Agro-Products, Chinese Academy of Agricultural Science, Beijing 100081,China.E-mail:yangshumingcaas@sina.com

* Jinzhen Zhang and shuming yang contributed equally to this work