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Determination of proline in meads using high performance liquid chromatography

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This work is focused on the determination of proline in 24 Czech mead samples using reversed-phase high performance liquid chromatography (RP-HPLC) coupled with fluorescence detector (operating at excitation wavelength λ_{ex} = 264 nm and emission wavelength λ_{em} = 497 nm). Sample pre-treatment including solid phase extraction of mead by Strata SCX SPE column and a derivatization of the extract acquired by dansyl chloride was performed and thoroughly optimised. Derivative of proline was separated using Ascentis Express C18 column (150 × 3 mm, 2.7 µm) in the combination with binary mobile phases that had consisted of 0.1% TEA (pH 3.0) and 100% acetonitrile. The method proposed could be used for evaluation of mead quality and its authenticity.

Keywords: Proline; Dansyl chloride; Meads; Derivatization; Extraction; Liquid chromatography

Introduction

Mead is a traditional alcoholic beverage formed by alcoholic fermentation of water-diluted honey using strains of yeast (usually *Saccharomyces cerevisiae*) [1]. Mead is a source of a wide range of substances, among which saccharides (especially fructose and glucose) are the most abundant. The composition of minor compounds, such as amino acids, organic acids, phenolic compounds, enzymes, vitamins, minerals etc., is variable and highly depending on the manufacturing practice and geographical and botanical origin of honey [2,3].

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Mead can be produced in the so-called cold or warm way and cooking of the honey-must prior to the yeast inoculation is the difference between these two. The preparation of un-boiled mead is technologically more challenging due to a higher probability of the occurrence of delayed, uncontrolled or stuck fermentation along with alterations of sensoric and quality parameters of the final product. Among others, these changes are caused by undesirable microbes naturally occurring in mead, which can be well eliminated by mead warming. On the other hand, honey-must heating leads to the formation of dangerous contaminant 5-hydroxy-methylfurfural (5-HMF) and losses of colour, flavour, and many health beneficial bioactive compounds, such as proteins, phenols, enzymes, vitamins and free amino acids [4–7].

Mead is very often falsified due to the lack of regulations and official control methods [3,8]. Analysis of phenols [9,10], organic acids [10], amino acids (AAs) [3,12–18], and saccharides profile [10,11], as well as the determination of the 5-HMF content [9–11] are commonly used methods for evaluation of the honey quality. Therefore, these methods could also be successfully applied in the assessment of mead samples. Proline is dominant amino acid in honey (mead, respectively), and its quantification is one of the most effective tools for the revelation of mead adulteration.

Reversed-phase high performance liquid chromatography (RP-HPLC) coupled to spectrophotometric (UV-VIS) or fluorescent detection is the most applied technique to perform the amino acids analysis. The direct determination of AAs is complicated due to the absence of fluorophore or chromophore in their structure and therefore, a transformation of AAs into the suitable derivative is necessary prior to the detection [19–21]. Many pre-column and post-column derivatization agents and methods has already been investigated, each with its advantages and disadvantages to be considered [19,22–26]. Despite all the available derivatization agents, our attention was paid to dansyl chloride (5-(dimethylamino) naphthalene-1-sulphonyl chloride; DNS-Cl). DNS-Cl is a very popular agent because of very stable derivatives with primary as well as secondary amines. On the other hand, DNS-Cl undergoes very easily hydrolysis and therefore, the derivatization procedure of choice must be thoroughly optimised [19,24,27,28]. General reaction of DNS-Cl with AA is shown by a scheme in Fig. 1.

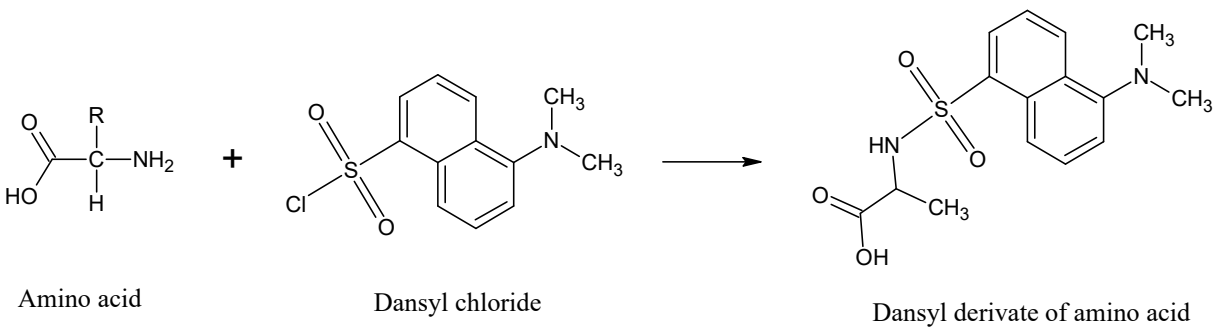

Fig. 1 Reaction pathway of the formation of DNS-AA derivatives

The aim of this study was to develop an analytical method for the proline determination in meads including the extraction and derivatization steps and chromatographic separation of DNS-AA derivatives. The procedure optimized was then applied to evaluate the quality of Czech meads.

Materials and methods

Chemicals and Reagents

Standard of L-proline (≥ 98%) together with some auxiliary derivatization components, such as lithium carbonate (≥ 99.0%) and sodium carbonate (≥ 99%) were purchased from Sigma-Aldrich (Steinheim, Germany); as well as methanol and acetonitrile (LC-MS purity) used for the preparation of mobile phases. Derivatization agent (DNS-Cl) was purchased from TCI (Toshima, Tokyo, Japan) and triethylamine (TEA; 99.8%) from Lach-Ner (Neratovice, Czech Republic). Hydrochloric acid (35%), formic acid (98%) and sodium hydroxide (98%) were purchased from Penta (Chudim, Czech Republic); all in p.a. grade. Demineralized water was prepared in a Milli-Q purification system (Merck Millipore, Darmstadt, Germany).

Analysed meads are listed in Table I. The samples were obtained from local Czech beekeepers or purchased in common stores. Samples were stored in the refrigerator and tempered at the room temperature before analysis.

Instrumentation

The HPLC system equipped with a LC-30AD binary gradient pump, a DGU-20A$_5$ degassing unit (both Shimadzu, Kyoto, Japan) and six-port valve with two-µL external loop (Valco-Vici, Schenkon, Switzerland) was coupled with an RF-20Axs fluorescence detector (Shimadzu). Ascentis Express C18 column (Supelco, Bellefonte, PA, USA) was situated in a LCO 102 column thermostat (Ecom, Prague, Czech Republic).

A vacuum unit Labobase SBC 860 (KnF, Stockholm, Sweden) together with a vacuum regulator Vacuubrand CVC 3000 (Vacuubrand, Wertheim, Germany) were connected to a 12-position Vacuum SPE Manifold (Labicom, Olomouc, Czech Republic) for solid-phase extraction (SPE) of the samples. SPE columns Supelco LC-SCX (1 mL, 100 mg; Supelco), Strata SCX (3 mL, 500 mg), Strata X-C (6 mL, 500 mg), Strata Screen-C (6 mL, 500 mg), Strata DSX-SCX, (6 mL, 500 mg; all Strata columns: Phenomenex, Torrance, CA, USA) were compared. The syringe PTFE filter (0.45 µm × 4 mm; Labicom) was used for filtration of derivatives before injection. Fluorat-02-Panorama spectrofluorometer (Lumex Instruments, St. Peterburg; Russian Federation) was used to measure excitation and emission spectra of the DNS-proline derivative.

Table 1 List of 24 analysed Czech meads, origin and type of production

Mead sample No.	Source, city	Preparation
1	Market	Un-boiled
2	Beekeeper, Pardubice	Un-boiled
3	Market	Un-boiled
4	Beekeeper, Nové Město nad Metují	Un-boiled
5	Beekeeper, Vamberk	Not specified
6	Beekeeper, Domažlice	Not specified
7	Market	Un-boiled
8	Beekeeper, Nové Město nad Metují	Un-boiled
9	Market	Un-boiled
10	Market	Not specified
11	Market	Un-boiled
12	Beekeeper, Nové Město nad Metují	Un-boiled
13	Beekeeper, Nové Město nad Metují	Un-boiled
14	Market	Un-boiled
15	Market	Boiled
16	Market	Not specified
17	Market	Un-boiled
18	Market	Not specified
19	Beekeeper, Domažlice	Un-boiled
20	Beekeeper, Pardubice	Un-boiled
21	Market	Un-boiled
22	Market	Boiled
23	Beekeeper, Domažlice	Un-boiled
24	Market	Boiled

Extraction procedure

Proline was isolated and concentrated using an SPE Strata SCX column. The extraction conditions were optimised as follows. The column was conditioned by 6 mL of methanol and 6 mL of water acidified by HCl (pH 3.0). Then, 10 mL of proline standard or 20 mL of mead sample (both adjusted with HCl to pH 1.9) were applied. Impurities were removed by 1 mL (for standard) or 5 mL (for sample) of HCl-acidified water (pH 3.0). Finally, 2 mL of 1M NaOH was used for elution of the retained AAs. The flow rate of the extraction process was approximately one drop per second.

Standards with three known concentrations of proline (89; 238; 298 mg L^{-1}) were used for determination of the extraction efficiency. Each concentration level was extracted five times and each extract injected three times after its derivatization. Then, extraction efficiency together with its standard deviation were calculated.

Derivatization procedure

A volume of 0.5 mL of extract was mixed with 5 mg of lithium carbonate and with 1 mL of 0.01M DNS-Cl in acetonitrile. Derivatization reaction was performed in the darkness. After 5 min, the solution was filtered through a 0.45 µm PTFE syringe filter and injected into the HPLC system.

Proline quantification

The calibration curve method was used for the determination of proline in meads. 10 mL of the individual calibration solutions in the concentration range of 108.2 mg L^{-1} to 238.0 mg L^{-1} were extracted, derivatized, analysed, when the dependence of the peak area on the proline concentration was subsequently plotted (see Fig. 2). Each calibration concentration level was prepared three times and analysed in three replicates by HPLC. Calibration equation of proline with the standard deviation (RSD) is also shown in Fig. 2.

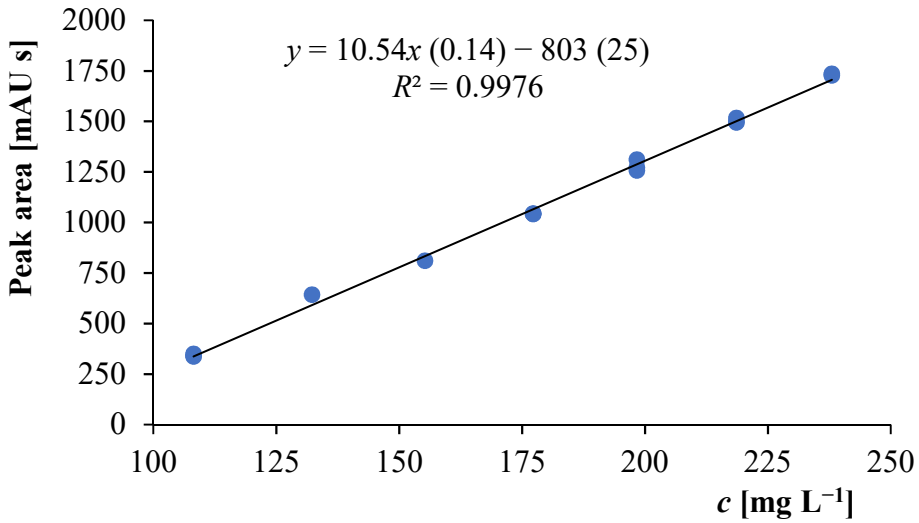

Fig. 2 Calibration curve of proline with corresponding calibration equation

HPLC analysis

HPLC separation of DNS-proline derivatives had required optimisation of several key experimental conditions. After doing so, the final separation was performed on Ascentis Express C18 (150 × 3 mm; 2.7 μm I.D.) column with the gradient elution by using binary mobile phase consisting of 0.1% TEA in water acidified by HCOOH to pH 3.0 (A) and 100% acetonitrile (B). Gradient program was as follows: 0 min – 40 % B, 2 min – 50 % B, 4 min – 100 % B; the flow rate being 0.8 mL min^{-1}, temperature 30 °C and the injection volume 2 μL. Excitation and emission wavelengths were set to λ_{ex} = 264 nm and λ_{em} = 497 nm, respectively. Software Clarity (DataAppex, Prague, Czech Republic) was used for data collection and evaluation.

Results and discussion

Optimisation of extraction procedure

SPE extraction was performed to eliminate the matrix effect and to increase the proline concentration. SPE columns, namely: Supelco LC-SCX, Strata SCX, Strata X-C, Strata Screen-C, and Strata DSX-SCX were tested in the terms of their recovery efficiencies determined by comparing the DNS-proline peak areas of the extracted and corresponding non-extracted standard of proline. Extraction recoveries were obtained in the range of 0.0–84.2 % and are shown in Table 2, together with confidence interval. Although all the columns had been suggested for AAs extraction, the zero recovery was found for the Supelco LC-SCX column. The best column was Strata SCX with the proline recovery of 84.2 %.

Table 2 Five investigated extraction columns (Supelco LC-SCX, Strata SCX, Strata X-C, Strata Screen-C, and Strata DSX-SCX) with corresponding values of extraction recoveries [%] and the confidence interval (α = 0.05)

SPE column	Efficiency [%]
Supelco LC-SCX	0.0 ± 0.0
Strata X-C	67.2 ± 2.5
Strata Screen-C	31.5 ± 1.6
Strata DSC-SCX	60.7 ± 2.3
Strata SCX	84.2 ± 4.8

Furthermore, other parameters as the extracted mead quantity (10–20 mL), initial pH of mead (1.6–3.0), and the type and amount of the elution solution (1–5 mL 0.01M NaOH or 1M NaOH, 8 % Na_2CO_3 or their mixtures) were optimised. The best results were obtained by extracting 20 mL of mead with pH 1.9 and elution with 2 mL of 1M NaOH.

Optimisation of derivatization procedure

Originally, the derivatization process was based on a relatively complicated procedure (for details, see [29,30]), where AAs react with DNS-Cl in the presence of lithium carbonate. The reaction mixture was heated in a water bath at 40 °C for 20 min without the presence of the light, and derivatives analysed after next 30 min equilibration.

This derivatization process was carefully optimised to simplify and speed up the desired reaction, and thus to reduce the sample-preparation time. Our attention was focused to find the best solvent for DNS-Cl, as well as the appropriate amount of derivatization agent needed for the reaction. Further, the necessity of incorporating the heating step and addition of lithium carbonate were optimised in term of the stability of the derivatives obtained.

Dansyl chloride undergoes very easily to hydrolysis and therefore, it is usually dissolved in methanol or acetonitrile. The efficiency of derivatization has been found evidently higher with the application of acetonitrile as the solvent of DNS-Cl. The pH value plays a key role in the proper course of the derivatization reaction, which should be kept at pH 9.5. However, sample extract is very alkaline (elution by 1M NaOH), the pH decrease is necessary to complete the derivatization reaction. For this purpose, an addition of lithium carbonate or sodium carbonate together with extract neutralization via varying volume of HCl (35–45 μL HCl) was performed and compared. The highest response of the detector was obtained with the addition 5 mg Li_2CO_3, which, among others, probably catalyses the derivatization reaction.

Further, it has been found that derivatization reaction proceeds immediately after the reagents are mixed. Thus, heating plays no role in the pathway of the reaction, and therefore, the derivatization can be performed at the room temperature. The derivatives obtained had remained unchanged for at least 24 hours.

Optimisation of HPLC analysis

The mobile phases used and their gradient profiles, together with the detection wavelengths, were assessed during RP-HPLC optimisation. The parameters, such as the type of organic component of the mobile phase (acetonitrile, methanol and

their mixture), pH of water component of the mobile phase (pH 2.5–3.5), effect of TEA addition (0–0.2 %) to the water component of the mobile phase, as well as various gradient slopes with different initial and final concentrations were tested. It was found that the best separation with symmetric and narrow peaks could be obtained with the gradient elution with binary mobile phase comprised of 0.1% TEA in water acidified to pH 3.0 (A) and 100% acetonitrile (B); the gradient program being as follows: 40–100 % B in 4 min.

The excitation and emission spectra of the prepared derivative were measured on a spectrofluorimeter to find the suitable wavelengths for the HPLC fluorimetric detector. DNS-proline derivative had shown the excitation maximum at 264 nm and emission maximum at 497 nm and therefore, detector was set to these values.

Optimised separation of the derivative of the proline standard solution (c = 115 mg L^{-1}) is depicted in Fig. 3.

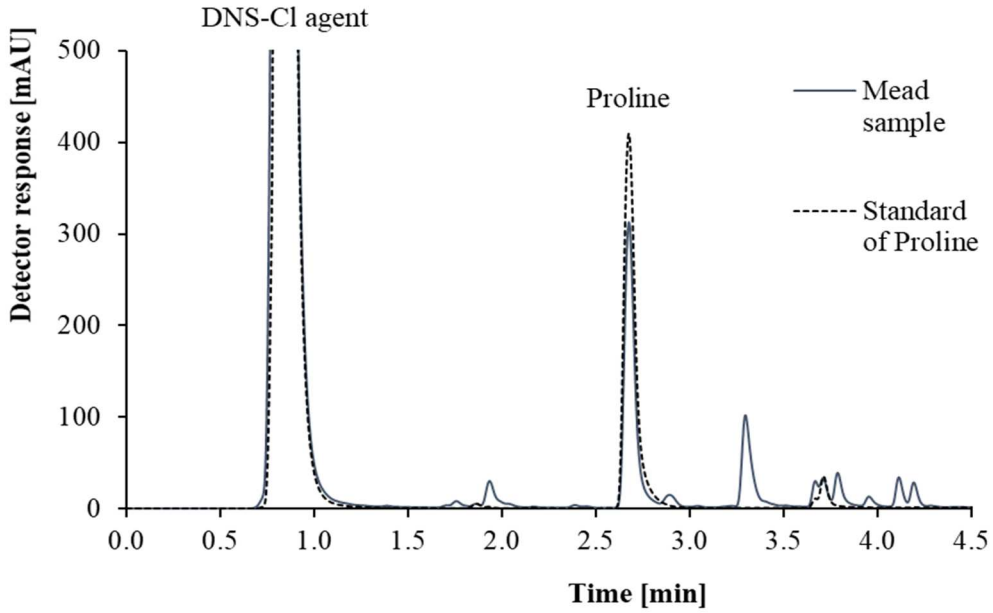

Fig. 3 Chromatogram of DNS-proline derivative (c = 115 mg L^{-1}) previously extracted on SPE Strata SCX column and chromatogram of derivatized mead extract (20 mL)

Column Ascentis Express C_{18} (150 mm × 3 mm × 2.7 µm); column temperature: 30 °C; mobile phase A: 0.1% TEA in water acidified by HCOOH to pH 3.0; mobile phase B: 100% acetonitrile; gradient program: 0 min – 40 % B, 2 min – 50 % B, 4 min – 100 % B; flow rate: 0.8 mL min^{-1}; detection wavelengths: λ_{ex} = 264 nm, λ_{em} = 497 nm; injection 2 µL

Data evaluation

In total, 24 Czech mead samples were extracted, derivatized, and analysed by optimised method to determine the proline content, and thus their quality and authenticity. Example of analysis of real mead sample is given in Fig. 3. The proline quantities found in all the samples are shown in Fig. 4.

Based on EC Regulation, the proline content in honey must be at least 180 mg kg^{-1} [8]. A recommended minimum of 280 kg honey should be used for the preparation of 1000 L of mead [31]. It means that the minimum limit should be 50 mg of proline in one liter of mead. As illustrated by Fig. 4, twenty analysed samples reached the minimum proline level. Samples No. 2, 4 and 13 contain the highest proline amount. These three meads had come from local beekeepers and were prepared by cold way. The high content of proline could be caused by additional sweetening with honey after their production. On the other hand, four samples, namely No. 9, 10, 15, and 18, did not reach the requirements for the minimal content of proline. All of these meads were obtained in the markets. As stated on the label of the respective mead bottles, sample No. 15 were prepared by warm (boiled) way, sample No. 9 by cold way and the preparation of samples No. 10 and 18 was not ascertained. The producers of these outliers evidently did not comply with the good manufacturing practice related to the requirements of minimal amount of used honey for the mead preparation and hence, these samples should be considered adulterated and of worse quality.

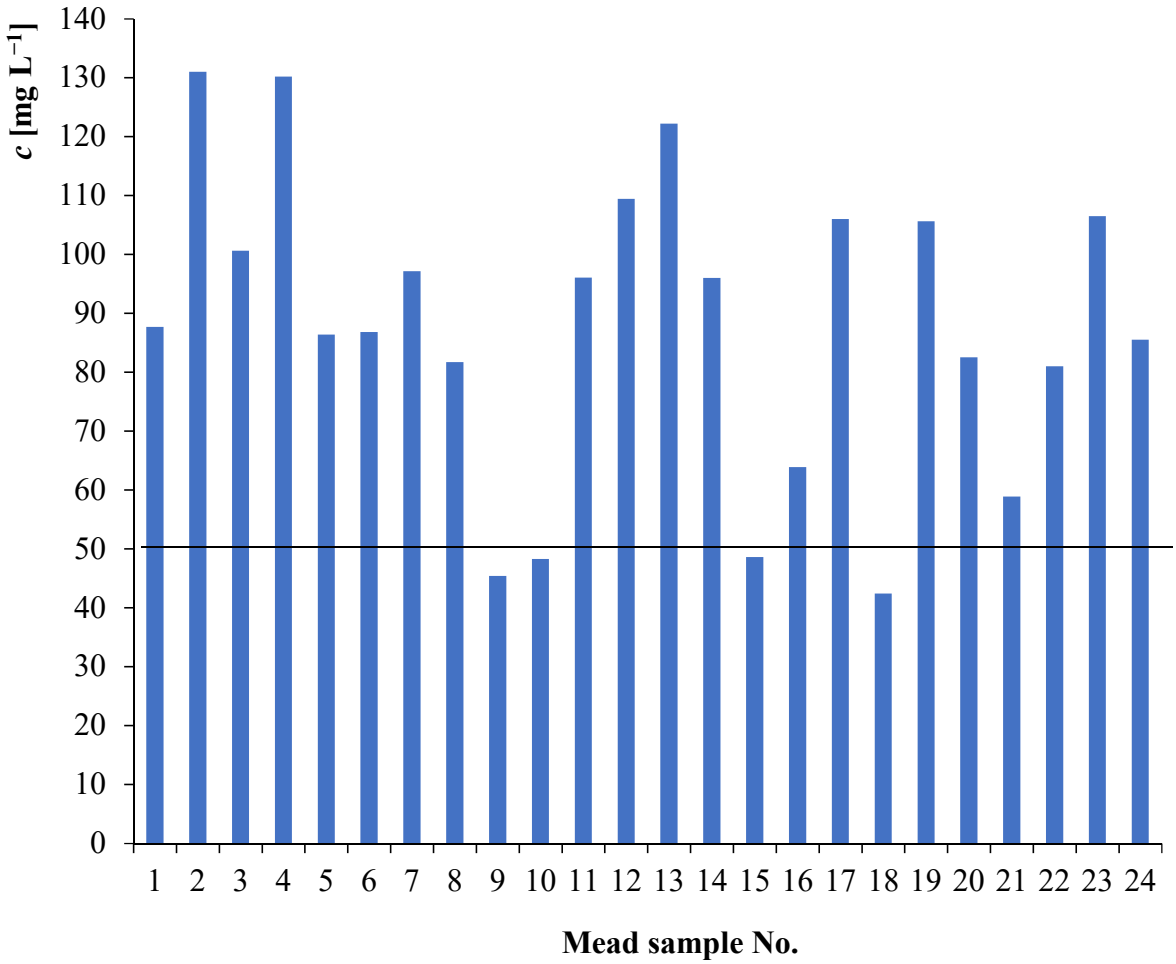

Fig. 4 Proline content in 24 Czech mead samples including minimum quantity line at 50 mg L^{-1}

Conclusions

In this work, the method for the determination of proline in 24 Czech meads purchased from local beekeepers and from markets was optimised. Proline was determined by high performance liquid chromatography coupled with the fluorescence detector after its previous SPE extraction by Strata SCX column and derivatization with dansyl chloride agent. The proline quantities ranged from 42.4 ± 3.1 mg L^{-1} to 131.0 ± 3.3 mg L^{-1} in the individual samples analysed. In general, commercial samples contained lower proline content than those purchased from beekeepers. The highest proline quantities were found in meads No. 2, 4 and 13, which had all been bought from beekeepers. On the other hand, four samples (No. 9, 10, 15 and 18) did not meet the required minimum concentration of proline given by the regulations [8,31]. All these samples were bought in local markets and the producers of these meads apparently did not follow the correct technological procedure.

Acknowledgement

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