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Scientific paper

Direct Determination of Kynurenic Acid with HPLC-MS/MS Method in Honey

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Abstract

Kynurenic acid (KYNA) has been attributed many beneficial properties, such as antioxidant, antiproliferative, anti-inflammatory, and anti-obesogenic, as it is believed to affect metabolism and weight gain. A rapid and simple HPLC-MS/MS method for the determination of kynurenic acid (KYNA) in honey has been developed. HPLC-MS/MS system allowed us to perform the analyzes without any special extraction or treatment of the samples. The study was carried out on different honeys: Chestnut (C), Linden (L), Acacia (A), Spruce (S), Silver Fir (SF), Forest (Fo) and Flower (F). The highest mean concentration, 682 μg/g, was determined for chestnut honey, making it one of the foods with the highest KYNA content.

Keywords: Kynurenic acid, honey, HPLC-MS/MS, SRM

1. Introduction

4-Hydroxyquinoline-2-carboxylic acid (structural formula shown in Figure 1), also known as kynurenic acid (KYNA), is a tryptophan metabolite, a byproduct of the kynurenine metabolic pathway, and was discovered by Liebig in 1853.[1] The kynurenine metabolic pathway is a process of dietary tryptophan metabolism and production of the cofactor nicotinamide adenine dinucleotide (NAD +). It is formed directly from kynurenine in a reaction catalyzed by kynurenine aminotransferases.[2] Studies and analyzes of KYNA have been performed on various samples. It has been detected in samples ranging from honeybee products, various plants, herbs and spices to cells and human and animal tissues and excretions showing anticonvulsant and neuroprotective activity.[3–15] In this study, the presence of KYNA in food and honeybee products was investigated. KYNA was found in all 37 tested samples of food and honeybee products. The highest concentration of KYNA was obtained from honeybee products' samples, propolis (9.6 nmol/g). Many properties were attributed to it, such as anti-ulcer, anti-inflammatory and anti-proliferation.[12,16–18] High antioxidant capacity and regulation of bacterial growth have also been observed along with properties of reducing hypermotility and antagonizing ionotropic glutamate receptors.[15,18–21]

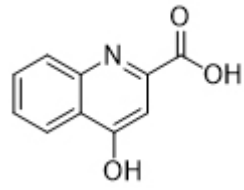

Figure 1: Structural formula of kynurenic acid (KYNA).

It has also been observed that KYNA concentration deviates from normal value if subject suffers from irritable bowel syndrome, Parkinson's disease, Huntington's disease, or multiple sclerosis, resulting in a decrease in concentration, while the opposite phenomenon has been observed in colon lesions such as adenomas or adenocarcinomas and inflammatory bowel disease, in which the concentration of KYNA is increased.[22–28]

There has also been a suspected association between lower KYNA levels and various types of mood disorders, a phenomenon which occurs primarily in women.[29] Consequently tryptophan and its metabolites such as kynurenine and kynurenic acid were investigated in human plasma.[30]

KYNA plays an important role as antagonist of ionotropic glutamate receptor and an agonist for the orphan G

protein-coupled receptor GPR35, which is found in the gastrointestinal tract and immune cells.[13,21] Mainly the positive properties in gastrointestinal tract and capability of decreasing hypermotility call for broader investigation of KYNA intake from food.[18,20] KYNA is found in many herbs, spices, and other remedies used to relieve digestive system problems.[10,11] For example common nettle or St. John's wort, both KYNA rich substances, are often used as remedies for reducing the symptoms of digestive system diseases.[10] This means that KYNA, among many other beneficial properties, may play an important role in digestion and also weight gain. KYNA has been suggested to be an anti-obesogenic compound which can influence weight gain. The concentration of KYNA has been studied in human breast milk and in baby formula, suggesting that a deficiency of KYNA in baby formula may lead to obesity of infants and children. This was further tested on rats, resulting in lower weight gain in rats postnatally fed with KYNA supplements compared to rats without it.[7]

In general, the methods developed so far require complex preparation of samples. This means mainly homogenization, centrifugation, and various extraction methods (such as Solid Phase Extraction – SPE) which potentially eliminate possible interferences. The method for determination of KYNA in potatoes and flour consisted of homogenization of samples and further centrifugation. KYNA was later extracted from the supernatant by SPE method using a cation exchange resin.[8] The same SPE method was used to determine KYNA in honey.[9] However, a RP-SPE cartridge filled with solid phase, was used in NMR and MS study of KYNA in plants.[4,31] Our goal was to simplify sample preparation.

Selectivity can also be improved in other ways, for example by using a liquid chromatography system coupled to a triple quadrupole mass spectrometry system in Selected Reaction Monitoring (SRM) mode to observe only the molecule of interest.[30,32,33]

All mentioned positive properties of KYNA and the need to optimize the preparation methods make honey of different botanical sources an interesting target for the study of KYNA content. Our main goal was developing a method avoiding all mentioned complex and not necessary preparations steps which resulted in cheaper and less time-consuming method for analysis of KYNA in honey matrix. Method was developed for HPLC-MS/MS system in SRM mode. An example of the method LC-MS/MS in SRM mode is shown in Figure 2. Newly developed method does not require any centrifugation or special extraction of the analyte and its selectivity does not depend on compounds fluorescence or UV light absorption so no other detector is necessary.[30,32,34] Analyzes were performed on Chestnut (C), Linden (L), Acacia (A), Spruce (S), Silver Fir (SF), Forest (Fo) and Flower (F) honey types.

2. Experimental

Honey

Different honey samples, in total 129, including chestnut (6), linden (14), spruce (2), acacia (20), silver fir (2), flower (59) and forest (26) honey, were obtained from local bee keepers and Medex (Medex d.o.o., Slovenia).

Sample preparation

KYNA (Sigma, USA) standard for calibration curve was dissolved in 0.1% NH_3 (Gram-Mol d.o.o., Republic of Croatia), as were the honey samples. 0.1 g of chestnut honey, 1 g of flower, forest, acacia and linden honey and 3 g of spruce and silver fir honey were separately dissolved in 0.1 % NH_3, mixed thoroughly, and filled to mark in 50 mL volumetric flask. The solution was filtered through nylon filter (pore size 0.45 μm) and transferred to vial.

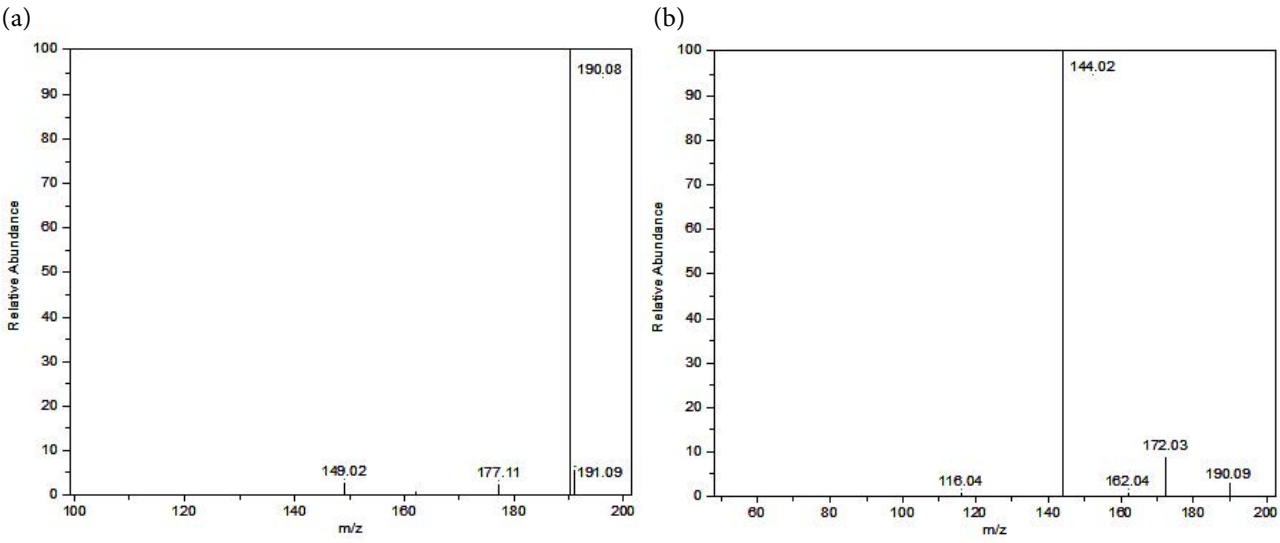

Figure 2: (***a***) MS spectrum of KYNA precursor ion; (***b***) MS spectrum of KYNA product ions.

For purposes of recovery determination L 131 and A 15 were prepared the same way and additionally spiked with KYNA standard, 105.5 mg and 9.1 mg, respectively.

HPLC-MS/MS Analysis

Analysis was developed for UHPLC system (Vanquish™ Flex UHPLC, Thermo Scientific™, USA) coupled with H-ESI-MS system (TSQ Quantis™ Triple Quadrupole Mass Spectrometer, Thermo Scientific™, USA).

Chromatographic conditions

Sampler compartment was thermostated at temperature 20 °C. Injection volume was 10 µL. Column (Kinetex® 2.6 µm C18 100 Å, LC Column 150 × 4.6 mm, Phenomenex Inc., USA) was thermostated at temperature 25 °C. Mobile phase A was 0.1% formic acid (Honeywell, USA) in ultrapure MilliQ water (Millipore, USA), mobile phase B was acetonitrile (Fisher Chemical, UK). Gradient method was developed at flow 0.7 mL/min. Elution was used as follows: time 1 min, 10% B; time 8 min, 80% B; time 11 min, 80% B; time 13 min, 10% B; time 18 min, 10% B.

Mass spectrometric conditions

A SRM method for H-ESI-MS was developed. H-ESI-MS was used in positive mode with spray voltage +190 V. Gases were optimized at following values: Sheath gas 8.55 L/min; Auxiliary gas 14.29 L/min; Sweep gas 1.5 L/min. Ion Transfer Tube Temperature was held at 350 °C and Vaporizer Temperature was held at 400 °C. SRM parameters for KYNA were: Precursor ion (m/z) 190.08; Product ion (m/z) 144.02; Collision energy 18 V.[30,32]

Determination of KYNA

The concentration and content of KYNA in honey was determined using method of calibration curve in concentration range from 0.01 mg/L to 20 mg/L.

3. Results and Discussion

This research was aimed at developing a HPLC-MS/MS method for determination of KYNA in honey matrix with application advantages such as avoiding the use of any special extraction method or other sample pretreatment consistently used laboratory practice so far. 129 honey samples were analyzed. Our study confirmed that KYNA is poorly soluble in acetonitrile and methanol.[34] The best MS-compatible solvent was determined to be 0.1 % ammonia; alkaline solvent improves solubility as well as stability of kynurenic acid.

During optimization of MS ion source conditions, we tested different ionization voltages. +190 V turned out to give the same, if not better, results as some higher voltages, used in experiments described in literature.[30,32] Easy ionization may be due to free electron pair on nitrogen while formic acid from mobile phase acts like an excellent proton donor.

Calibration curve, LOD, LOQ

Concentrations were determined by a calibration curve. 3 calibration curves, covering the entire linearity range from 0.01 mg/L to 20 mg/L, were used. Equation for calibration curve in range (0.01 – 0.1) mg/L was $y = 2*10^7x + 14246$ with coefficient of determination $R^2 = 0.9987$. Equation for calibration curve in range (0.1–1) mg/L was $y = 2*10^7x + 282943$ with coefficient of determination $R^2 = 0.9994$. Equation for calibration curve in range (1–20) mg/L was $y = 2*10^7 + 8*10^6$ with coefficient of determination $R^2 = 0.9957$. LOQ and LOD were determined experimentally analyzing low concentration standard solutions. LOQ was determined to be 0.01 mg/L (S/N = 10), while LOD was determined to be 0.001 mg/L (S/N = 3), which would give a KYNA content of 0.5 µg/g and 0.05 µg/g, respectively, given the weight of the honey sample was 1 g.

Repeatability, stability and recovery

Repeatability, shown in Table 1, was investigated for each honey type. Three parallel samples of each representative honey type were prepared. Relative standard deviation (RSD) of the peak area was calculated.

Table 1: Repeatability of each honey type.

SAMPLE	RSD [%]
Chestnut	2.9
Spruce	2.2
Silver Fir	2.8
Linden	1.2
Acacia	2.8
Forest	4.0
Flower	1.0

The stability of three KYNA standard solutions at concentrations of 0.05 mg/L, 0.5 mg/L, and 10 mg/L at 20 °C was studied over a 21-day period. They were all found to be stable over this period with relative standard deviations of instrument response over time of 11.0%, 9.6% and 8.7%, respectively. The stability of the standard solution with a concentration of 0.5 mg/L over time is presented in Figure 3.

For purposes of recovery determination L 131 and A 15 were spiked with KYNA standard. The recoveries for L 131 and A 15 were 106% and 113%, respective-

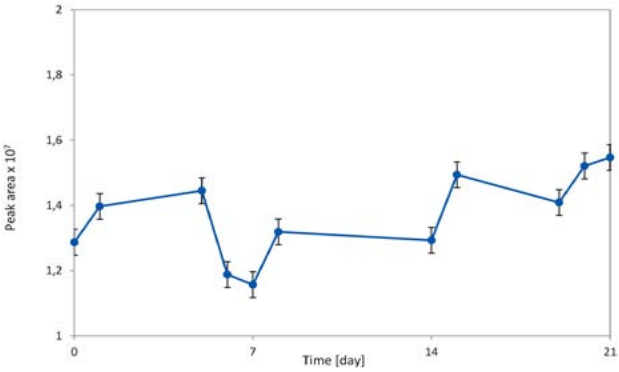

Figure 3: Stability of standard solution with concentration 0.5 mg/L of KYNA. Standard deviations were determined with three consecutive parallel determinations.

131 sample and L 131 spiked with KYNA standard can be seen in Figure 4.

Concentration in honey

As shown in Figure 5 concentration of KYNA is the highest in chestnut honey in range (327.8–1015.7) μg/g followed by linden honey in general range (24.6–188.7) μg/g. Next, in general order is spruce honey in range (8.0–8.9) μg/g followed by acacia honey in general range (0.7–5.3) μg/g. Somewhere in between acacia honey range is silver fir honey with concentration range (1.4–2.2) μg/g. Results of spruce, acacia and silver fir honey are presented in Figure 6.

As expected the range of mixed honey samples, such as flower honey and forest honey, varies from low to high concen-

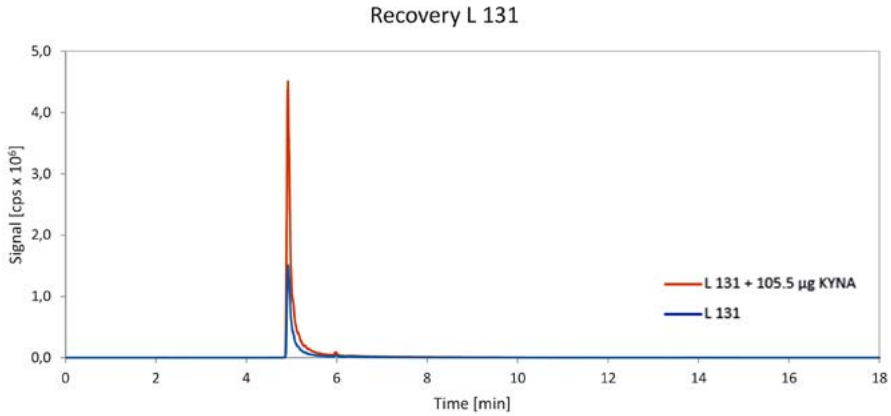

Figure 4: SRM chromatogram of Linden 131 sample and Linden 131 spiked with KYNA standard.

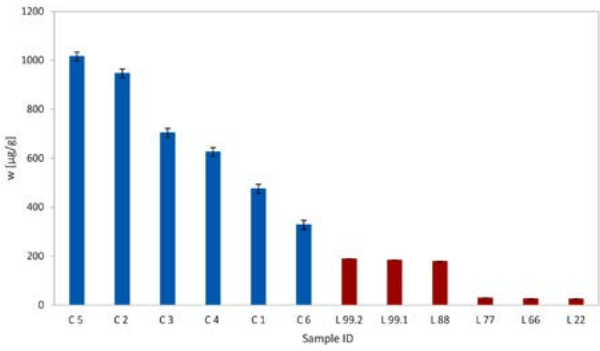

Figure 5: KYNA concentration in all chestnut (C) honeys (samples C 1–6) and three samples of linden (L) honey with maximal concentration (sequence label L 99.2, L 99.1 and L 88) and three samples with minimal concentration (sequence label L 77, L 66 and L 22) of KYNA. Standard deviations were determined on three consecutive parallel determinations.

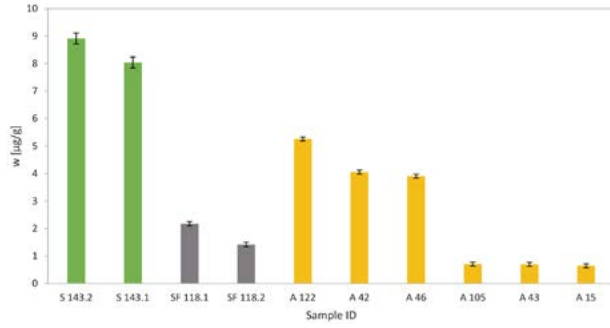

Figure 6: KYNA concentration in both samples of spruce (sequence label S 143.2 and S 143.1) and silver fir (sequence label S 118.1 and S 118.2) honeys and three samples of acacia (A) honey with maximal concentration (sequence label A 122, A 42 and A 46) and three samples with minimal concentration (sequence label A 105, A 43 and A 15) of KYNA. Standard deviations were determined on three consecutive parallel determinations.

ly. These specific samples were selected based on their KYNA content, which was moderate in L 131 (106.2 μg/g) and low in A 15 (0.7 μg/g), making them perfect for the recovery study. L 131 was spiked with 105.5 μg and A 15 was spiked with 9.1 μg of KYNA. Chromatogram of L

trations with no observable order. Therefore, the concentration of KYNA in forest honey and flower honey samples ranged from (0.8–397.7) μg/g and (1.4–194.2) μg/g, respectively.

The content of KYNA is the highest in chestnut honey then followed by linden honey and others. It can

be noticed that concentrations of KYNA in some acacia honey samples are higher than its general range, as the concentration of KYNA in some linden honey samples are lower than its general range which could be explained as presence of some other type of honey which increases or decreases KYNA concentration. Reason for this could be mixing honey with honey of different type, intentional dilution of honey or heterogeneity of honey bee apiary area.[35,36]

As for flower and forest honey it was expected to have wide concentration range of KYNA. Besides the mentioned reasons, for out of general range concentrations, explanation of this phenomenon, could be wide range of botanical sources of honey as this is not honey from one specific plant. High concentration of KYNA in some forest and flower honey may suggest a greater share of chestnut honey or even linden honey.

Comparison with other KYNA rich substances

Similar study was also conducted on honeys from different countries where it was also evident that chestnut honey has the highest KYNA content. Concentration range was determined to be (103–141) μg/g or in another study (129–601) μg/g for chestnut honey, (0.177–0.391) μg/g for linden honey and (0.093–0.124) μg/g for flower honey.[9,33] These results somehow overlap with our results for chestnut honey, but are significantly lower in comparison with our results for linden and flower honey. From another set of results containing flower honey it is evident that the determined concentration of KYNA is 0.878 μg/g.[3] This may suggest that concentration of KYNA may also be dependent on soil, environment or fertilizer.[10]

Concentration of KYNA in honey samples is also high in comparison with other food. In other studies potato was suggested as food with high KYNA content where concentration varies (0.239–3.240) μg/g dry weight which quite coincides with concentration range of KYNA in acacia honeys.[8] There are also some herbs and spices with high amount of KYNA with basil as one of the most prominent representatives with concentration 14.08 μg/g which positions basil in between linden and spruce honey, but not even close the amount of KYNA in chestnut honey.[11]

Based on our results we can say that chestnut and linden honey are KYNA rich substances. Since the concentration of KYNA is considerably high in chestnut honey the source of it must be a part of chestnut tree. Research on chestnut tree parts (flower, peeled chestnut, nectar, pollen, …) suggest that the source of KYNA is nectar of male flowers, since female flowers do not produce nectar.[9,37] KYNA in chestnut nectar is also observed in another study where the content of honey bee's stomach of honey bee collecting in chestnut wood was investigated.[38]

4. Conclusions

Our main objective was to develop an optimal method for preparation and analysis of KYNA in honey samples resulting in a fast and simple method with very few steps, avoiding any kind of extraction or other special pretreatment of samples that have been used by most so far. HPLC-MS method, where MS detector was used in SRM mode, was developed. Main focus was the optimization of SRM parameters allowing us to perform a selective analysis of KYNA within the untreated samples (except dilution and filtration). Mainly the spray voltage and collision energy needed to be optimized for better selectivity and flows of sheath, auxiliary and sweep gasses for better limit of detection. Interestingly the spray voltage in positive was determined to be only 190 V, which can be attributed to using the correct mobile phase and other MS parameters. The fragmentation of precursor ion was also investigated, since there is many product ions, and it was determined that the fragmentation to the product ion (m/z) 144.02 is the most suitable for selective determination; collision energy for that reaction was optimized and determined at 18 V. Honeys of chestnut and linden botanical species were found to be rich in KYNA, with average contents of 682 mg/g and 85 mg/g, respectively, followed by spruce (8.5 mg/g), acacia (2.2 mg/g), and silver fir (1.8 mg/g) honeys. Forest honey (0.8–397.7 mg/g) and flower honey (1.4–194.2 mg/g) show a very wide range of concentrations, which could be attributed to them being honey of various floral sources or heterogeneity of apiary area; a higher KYNA content could also suggest presence of chestnut or even linden honey. These results could lead chestnut or even linden honey under consideration as food supplement for relieving of digestive problems or influencing digestion and body weight. In addition, the results could as well set values for KYNA content to detect altered honeys.

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Povzetek

Kinurenski kislini (KYNA) pripisujejo številne koristne lastnosti, kot so antioksidativne, antiproliferativne, protivnetne in anti-obesogene značilnosti. S svojim delovanjem naj bi vplivala na metabolizem in s tem na uravnavanje telesne mase. Razvili smo hitro, enostavno in zanesljivo HPLC-MS/MS metodo za določanje kinurenske kisline (KYNA) v medu. Sistem HPLC-MS/MS nam je omogočil izvedbo analiz brez posebne ekstrakcije ali obdelave vzorcev. V raziskavi smo analizirali med različnih botaničnih vrst, in sicer kostanjev (C), lipov (L), akacijev (A), smrekov (S), hojev (SF), gozdni (Fo) in cvetlični (F) med. Najvišjo povprečno koncentracijo kinurenske kisline (682 μg/g) smo določili v kostanjevem medu, kar ga uvršča med živila z najvišjo vsebnostjo KYNA.