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Development of modern analytical methods for analysis of biologically active substances

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Abstract

The dissertation deals with the development of analytical methods for the determination of selected biologically active compounds in three different matrices. The phenolic profile in Italian extra virgin oils and the amino acid profile in Czech mead were elucidated by liquid chromatography. The last type of samples were energy drinks, in which the amount of disputable β-amino acid taurine was monitored. The obtained results can be used for evaluation of the quality and authenticity of the products.

Abstrakt

Disertační práce se zabývá vývojem analytických metod pro stanovení vybraných biologicky aktivních sloučenin ve třech rozdílných matricích. Pomocí kapalinové chromatografie byl objasněn fenolický profil v italských extra panenských olejích a aminokyselinový profil v českých medovinách. Posledním typem vzorků byly energetické nápoje, v nichž bylo sledováno množství diskutabilní β-aminokyseliny taurinu. Získané výsledky mohou sloužit k hodnocení kvality a autenticity daných produktů.

Keywords

Phenolic compounds, amino acids, extra virgin olive oils, meads, energy drinks, liquid chromatography

Klíčová slova

Fenolické látky, aminokyseliny, extra panenské olivové oleje, medoviny, energetické nápoje, kapalinová chromatografie

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1 Phenolic Compounds in Extra Virgin Olive Oil

1.1 Introduction

Extra virgin olive oil (EVOO) is a non-polar liquid phase formed after the milling of fresh fruits of *Olea europea*. EVOO is obtained exclusively by cold pressing of olive paste, without any additives and other manipulations except decantation, centrifugation, and filtration [1–3].

Triacylglycerols (99 %), diacylglycerols and free fatty acids are the major oil compounds [4–7]. Perfect balance of these components together with other minor bioactive oil substances such as phenols, vitamins, sterols, and pigments are responsible for the EVOO health benefits [8–10]. Although, phenolic compounds (PPs) are only minor oil components (50–1500 mg/kg), these are very valuable bioactive substances because they bring many advantageous properties to the EVOO and play an important role in its organoleptic properties [11]. EVOO contains more than 36 structurally different phenolic compounds. Except the major secoiridoids, olive oil comprises also phenolic acids, phenol alcohols, flavonoids, lignans, and aldehydes [10,12–17].

Climatic conditions, olive-growing soil quality and composition, altitude and latitude, technological aspects, olive tree cultivar, olive fruits maturity and whole oil production process significantly influence EVOO composition and hence its quality and sensory, nutritional, physical and chemical properties [10,12,17–25].

Consequently, many analytical methods have been developed for the differentiation and classification of olive oils, which are usually focused on the determination of various organic compounds [26,27] such as fatty acids, triglycerides [28–30], minerals [25], free sterols, alcohols, tocopherols, hydrocarbons, pigments or just PPs [8,28,30]. PP profile and content could provide information about the geographical origin, technological practice, and other factors, which could help to distinguish the authenticity of the olive oils [31,32].

Reversed-phase high-performance liquid chromatography (RP-HPLC) combined with mass spectrometric (MS), spectrophotometric (UV/VIS), diode array (DAD), fluorescence or electrochemical detection is frequently used technique for analysis of PPs in olive oils. However, solid-phase or liquid-liquid (micro)extraction is always necessary prior the separation [9,12,23,33–35]. Few numbers of studies deal with the identification and quantification of PPs using gas chromatography, nuclear magnetic resonance (NMR), or infrared spectroscopy [2,25,30,32,36–39].

The aim of this work was to evaluate EVOO samples produced in different part of Italy in the terms of the composition and total phenol content using multivariate statistical methods. The second approach of this work was the comparison of two complementary analytical methods, HPLC and NMR [40,41].

1.2 Results and discussion

1.2.1 Optimisation of PPs extraction and separation

At first, appropriate extraction procedure of PPs was investigated. For this purpose, five different extraction methods were compared: miniaturised liquid-liquid extraction (LLE) according to Impellizzeri and Lin [42], original method of Montedoro et al. [43] and its miniaturisation form, SPE according to De Nino et al. [44] and LLE following the group of Ricciutelli et al. [45]. The attention was given to the initial amount of olive oil for extraction as well as the kind and amount of needed solvents, time of extraction procedure and drying process, and gained profile and quantity of identified compounds. The slightly modified extraction procedure of Ricciutelli et al. [45] was demonstrably better than the other investigated methods. This strategy used only 1.0 g of olive oil dissolved in 1 mL of *n*-hexane and the extraction was performed four times by 1 mL of methanol/water mixture. Polar phase was collected, evaporated, reconstituted with 0.5 mL of methanol (MeOH), and analysed [40].

Separation conditions of extracted PPs by RP-HPLC-DAD/MS instrument were optimised. Different kinds of stationary phase (SF) as well as conditions of starting concentration of organic part of mobile phase (MF), slopes of gradient elution as well linear or stepwise gradient elution were considered as optimised parameters. Optimised separation included Ascentis Express C18 (150 × 4.6 mm; 2.7 μm) column combined with 0.1% formic acid in water and 0.1% formic acid in acetonitrile (ACN) working in the gradient mode as follows: 0 min, 10 % B; 4 min, 35 % B; 12 min, 47 % B; 12.5 min, 60 % B; 16 min, 75 % B; and 21 min, 100 % B [40].

1.2.2 Method validation

The chromatographic method was validated by determining the parameters of linearity, intraday and interday repeatability, recovery of the extraction, limits of detection (LODs) and limits of quantification (LOQs) for 6 PPs standards, namely gallic acid, hydroxytyrosol, tyrosol, caffeic acid, ferulic acid, oleuropein, luteolin, and apigenin. Interday repeatability was slightly higher than intraday, however, all repeatability values showed very satisfactory results with relative standard deviation RSD ≤ 1 %. Recoveries were determined in the range of 84.3–99.5 %. Coefficients of linearity were sufficient and varied in the range of 0.9994–0.9999. The LOD values ranged from 4.4 to 39.9 mg/kg, and the LOQ values ranged from 17.5 to 133.0 mg/kg [40].

1.2.3 Quantity of PPs in the EVOOs

Olive oil is known for the presence of many derivatives, isomers, and other PPs forms [18]. Forty selected PPs, namely hydroxytyrosol (HTY), tyrosol (TY), tyrosol glucoside (TY-Glu), four elenolic acid isomers (EA is.), three verbascoside isomers (VER is.), apigenin (AP), luteolin (LU), oleacein (HTY-EDA), eleven oleuropein aglycone isomers (HTY-EA der.), three oleuropein isomers (HTY-EA is.), nine ligstroside aglycone isomers (TY-EA is.), three isomers of ligstroside aglycone

derivatives (TY-EA der.) and acetoxypinoresinol (AOP) were determined in 68 EVOO samples (Fig. 1) coming from 9 Italian regions: Apulia, Calabria, Campania, Liguria, Lazio, Tuscany, Umbria, Sicily, and Garda composed of Lombardy, Veneto, and Trentino-Alto Adige, using optimised HPLC-DAD/MS method [40]. PPs were identified according to the UV and mass spectra and information from the literature [16,35,36,39]. Due to the commercial unavailability of standards of individual isomers, the quantification was performed by calibration curve method using structurally similar analogues.

The derivatives and isomers of TY-EA and HTY-EA were the most significant compounds with the highest contribution to the total phenol content (TPC). On the other hand, VER isomers and AP were the minor compounds [41].

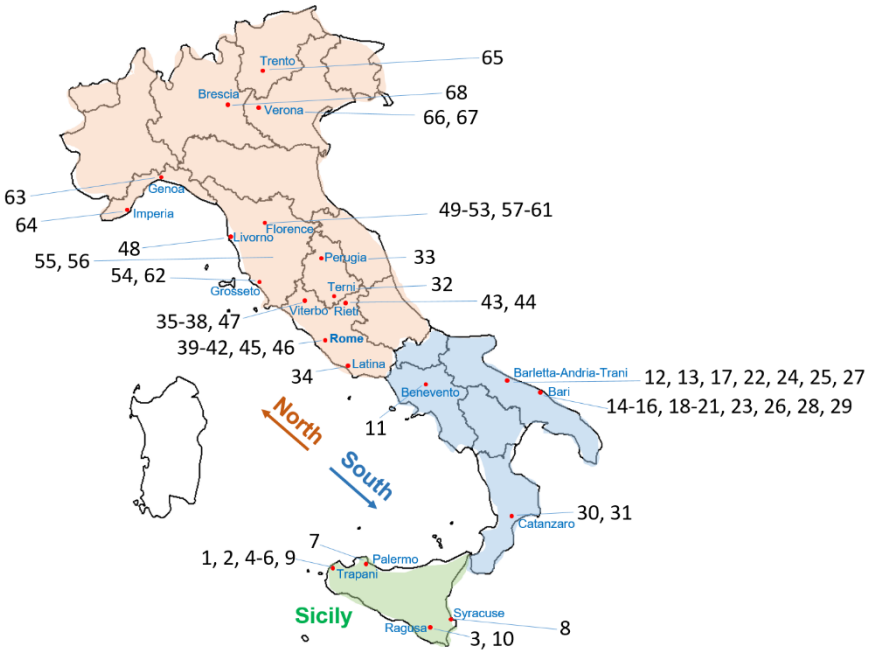

Fig. 1 Map of the Italian regions with the sample representation.

TPC in studied olive oil samples is shown in the Fig. 2. Samples from Apulia and Tuscany contained the highest TPC with the average about 700 mg/kg from which the samples 21 (Apulia) and 61 (Tuscany) were the richest EVOOs regarding the phenol content (1220 mg/kg and 1110 mg/kg, respectively). The pedoclimatic aspects, which are related to the geographic origin, together with the technological aspects are the main parameters influencing the TPC in EVOO samples. On the other hand, the botanical origin influences mainly the phenolic profile and the contribution to the TPC is lower [46–49]. The samples from northern Italian regions such as Garda and Liguria, together with samples from Sicily contained low amount of phenolic substances, with the average of 355 mg/kg. The lowest TPC was observed in the samples 3 and 8 (260 mg/kg and 257 mg/kg, respectively), both obtained from *Tonda Iblea* olives coming from the southernmost part of Sicily [41].

Since olive varieties are in most cases bound to a specific location (i.e. Sicilian olive cultivars do not grow in other parts of Italy), we cannot unambiguously claim, if the TPC is more related to the geographical origin or to the olive cultivars, which is nowadays frequently in dispute. This situation especially fits to Italy, where more than 500 of olive cultivars occur. All cultivars are not suitable for each environment; therefore, the different PPs amount and composition could be obtained from the same cultivar grown in different locations [47].

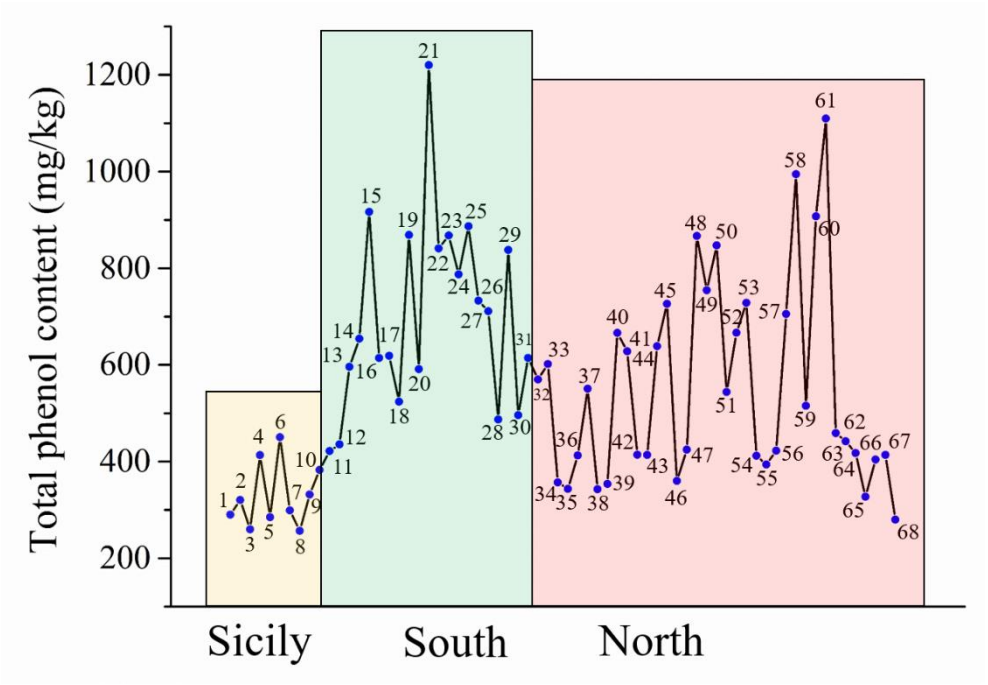

Fig. 2 Total phenol content [mg/kg] in all analysed EVOO samples, which are sorted by geographical origin.

1.2.4 Multivariate statistical analysis

Obtained data set was subjected to factor analysis (FA), principle component analysis (PCA) and linear discriminant analysis (DA) for the EVOOs evaluation [41].

Factor analysis

FA finds the underlying structure in a data matrix and allows the visualization of the variance between the olive oils. The Fig. 3 brings factor loadings after varimax rotation. From the Fig. 3 it is evident that derivatives of HTY-EA and TY-EA are useful for the factor 1 description, because these are located close to -1 on the axis of factor 1 and close to 0 on the axis of the factor 2. Derivatives and isomers of HTY-EA and TY-EA most contribute to the TPC of the EVOOs; therefore, the factor 1 relates to the phenol content. The objects EA, HTY-EDA and HTY suit the factor 2 description because they are located close to 1 on the axis of the factor 2 and close to 0 on the axis of factor 1. It was found [50], that EA isomers and HTY-EA derivatives are indicators

of the olives´ ripeness. The EA content increases with olives ripening while the HTY-EA content decreases [48,49]. Thus, the factor 2 is connected to the ripeness of the olives.

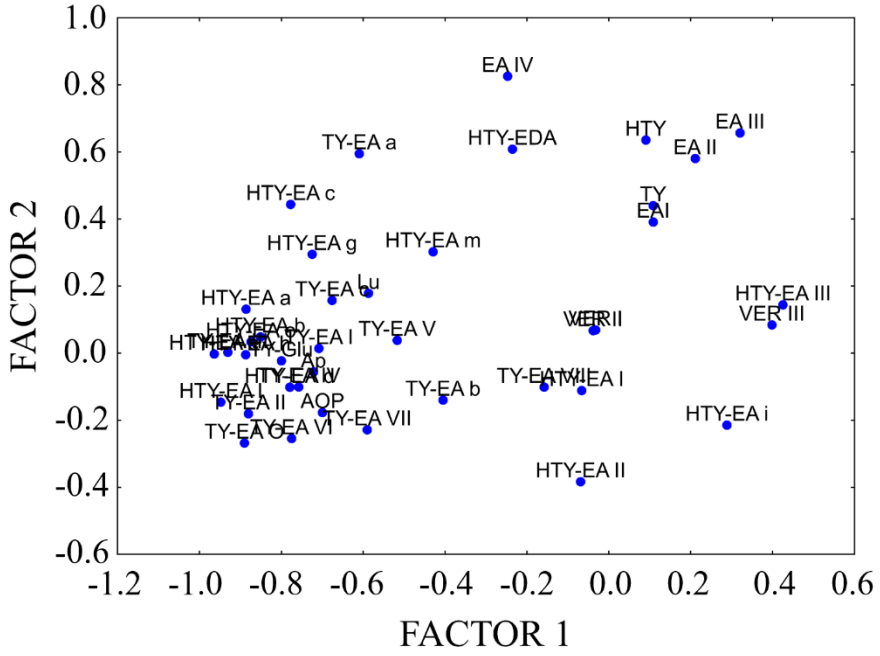

Fig. 3 The factor loadings plot after normalized varimax rotation (STATISTICA, StatSoft).

Using FA, the EVOO samples could be divided into two groups (Fig. 4). The samples with high TPC (mainly from Apulia, Tuscany, and Lazio) are located on the left part of the factor score (group A) and the samples with low TPC (mainly from northern Italian regions and Sicily) are located on the right part of the plot (group B). Simultaneously, the samples located on the top of the factor score contain high amount of EA and lower amount of HTY-EA which indicate that the olive oils could be prepared from the over-ripened olives (Fig. 4).

Further, five outliers (samples 1, 21, 49, 57 and 61) were found in the factor score (Fig. 4). Samples 21 and 61 showed the highest TPC from all analysed olive oils, thus, these are located to the far left. Samples 49 and 57, which are located on the top of factor score, contain enormously high amount of EA (and lower content of HTY-EA) compared with other EVOOs. These oils were probably produced from over-ripened olives; however, the TPC seems to be reasonable. The last outlier was the sample 1 observed in the upper corner far right of the Fig. 4. This sample contained very high amount of EA while its TPC was deeply below the mean value. Consequently, this sample was probably prepared from over-ripened olives and moreover, the olives could be grown, or the oil could be processed and stored in inappropriate conditions.

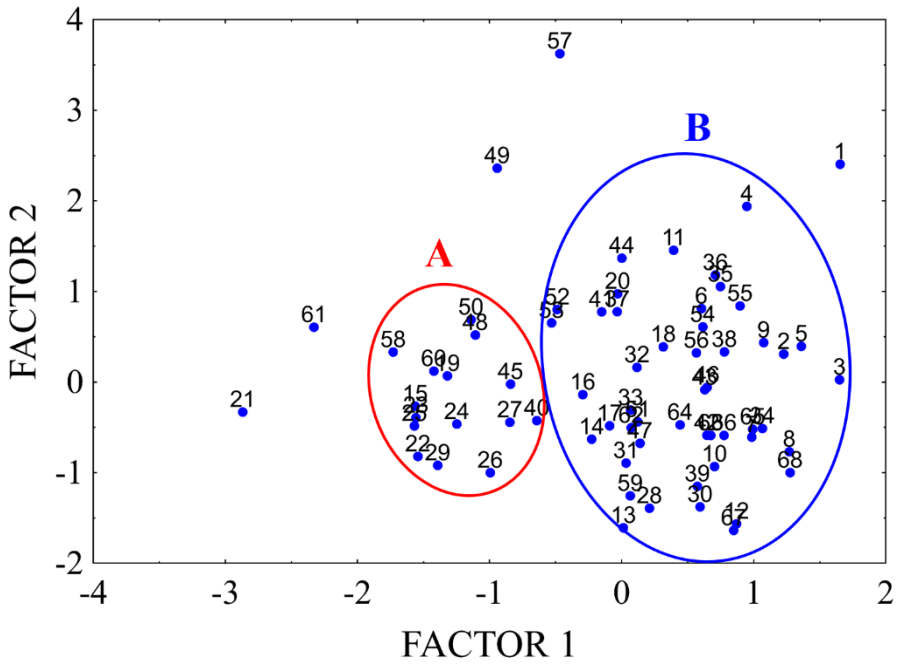

Fig. 4 The factor scatterplot of the first and the second factor (STATISTICA, StatSoft).

Discriminant analysis

EVOO samples were divided into three groups (A–C) according to their geographical origin using DA (Fig. 5). The group A (Fig. 5) includes the EVOOs from northern part of Italy (samples 32–68). Sample 40 is a bit far from the others, which could be caused by higher amounts of LU and AP. The chemical composition of these EVOOs is similar and olive cultivars are typical for northern Italian regions (*Casaliva*, *Leccino*, *Garda Orientale*, *Moraiolo*, *Toscano*, *Ca*rboncella, *Lavagnina*, *Taggiasca*, *Chianti classico*, *Olivastra Seggianese* and their mixtures). The group B (Fig. 5) comprises Italian southern EVOOs (samples 11–31) mainly prepared from *Coratina* olive cultivar. These samples came from the coast of the Adriatic Sea (all Apulian samples; 12–30), except the sample 11 from inland part of Campania, and then samples 30 and 31 coming from the coast of colder Ionian Sea. Just these two samples (30 and 31) could be considered as slight outliers of the group B which may be caused by the geographical position (the southernmost Italy) with different sea type and special olive cultivars (*Carolea*, *Lametia*). Apulian sample 29 from *Coratina* cultivar was located a little bit far from the rest of the samples of the group B. It could be caused by technological aspects of cultivation (olives growth, irrigation, plant health condition, olives harvest and collection) and processing (mainly crushing, malaxation, pressure of extraction, centrifugation, and storage) [46–49,51]. The C group includes Sicilian EVOOs which are made from the typical Sicilian olive cultivars *Nocellara del Belice* and *Tonda Iblea*. The C group (Fig. 5) is not clustered as good as previous two groups due to smaller number of the samples coming from this part of Italy. However, the main difference between these samples and the samples from the other two groups is in the position of Root 2 (Fig. 5), probably due to different climatic island condition.

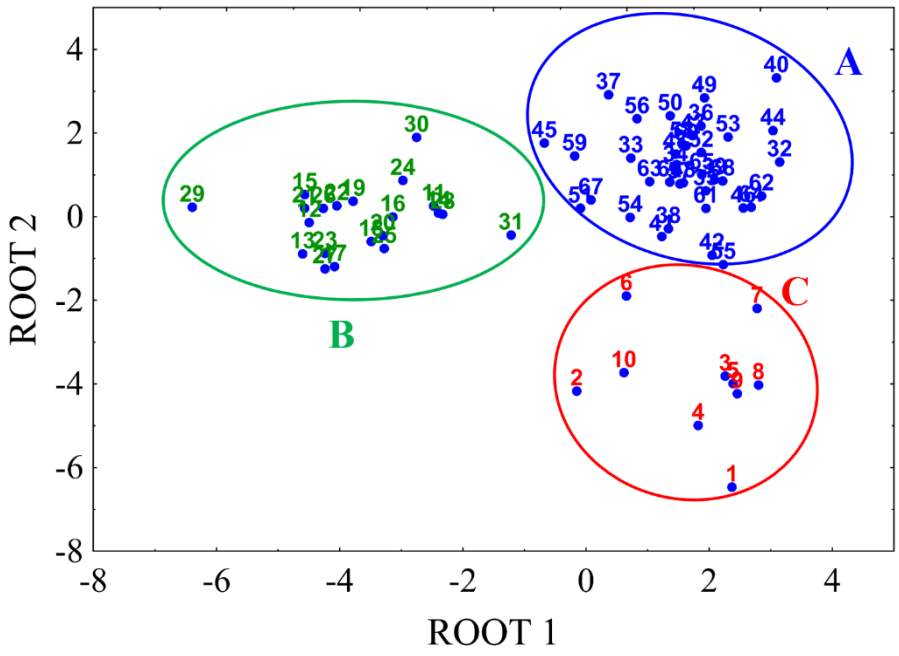

Fig. 5 Scatterplot of the linear discriminant score. The EVOO samples are divided into three clusters according to their geographical origin: Northern Italy – group A, Southern Italy – group B, and Sicily – group C (STATISTICA, StatSoft).

1.2.5 Comparison HPLC and NMR method

Thirty-two selected EVOOs were simultaneously analysed by NMR method without any sample pre-treatment or previous separation, targeted on compounds bearing the aldehydic chemical group [40]. HPLC and NMR spectra provide almost unique fingerprints for any sample. HPLC-DAD analysis enabled the quantification of 13 different compounds (40 individuals in total); while NMR experiments led to the quantification of 4 chemical species (6 individuals in total). Three substrates: HTY-EDA, TY-EA, and HTY-EA were detectable and quantifiable by both techniques. All the 32 samples have shown a consistent HPLC-DAD and NMR profile for TY-EA and HTY-EA derivates (Fig. 6).

On the other hand, there was a very enormous quantification mismatch of HTY-EDA, where the HPLC-DAD quantification provided lower values of 1 order of magnitude. According to the already published data, the NMR quantification of HTY-EDA is in the same order of magnitude referred to the other secoiridoid analogues [52,53]. This dramatic discrepancy is caused by a systematic underestimation when traditional HPLC-DAD is used due to a partial peak integration (the reconstructed ion chromatogram of HTY-EDA shows an unusual profile unveiling the presence of overlapped metastable forms). This is supported by Ammar et al. [54], showing a similar profile of HTY-EDA by HPLC-MS/MS and reporting its quantification values of at least 1 order of magnitude higher than that for the other HPLC research studies. Thus, NMR analysis is useful and even preferred in some cases.

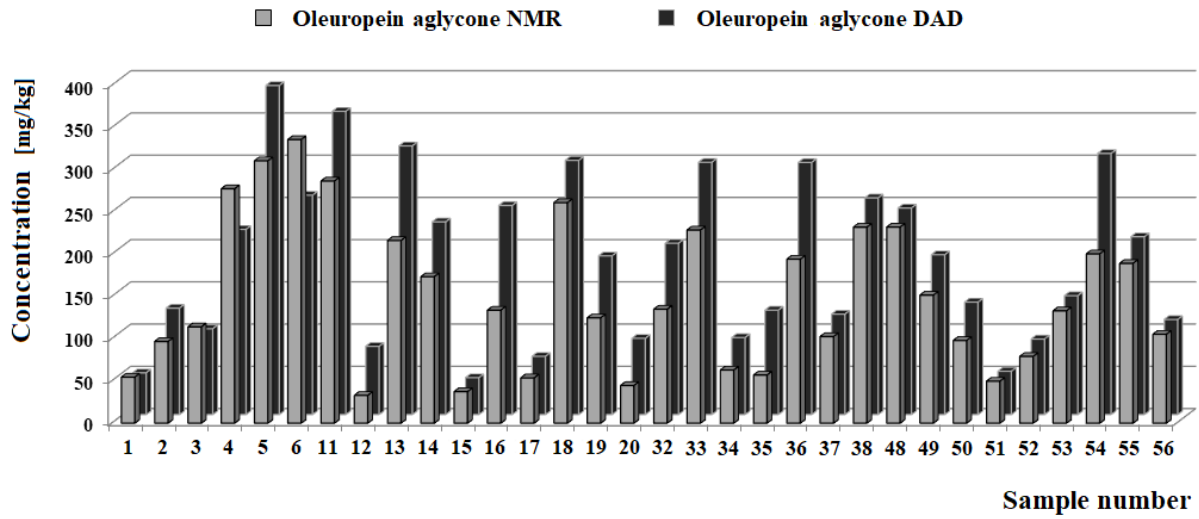

Fig. 6 Amount of the oleuropein aglycone in 32 EVOO samples acquired by NMR and HPLC analysis.

1.3 Conclusion

In the present study, PPs in 68 Italian EVOOs were characterised by optimised HPLC-DAD/MS method. Then, the data set was subjected to multivariate statistical analysis (FA, PCA, and DA) to distinguish the EVOOs based on the different parameters such as geographical origin, ripeness of the olives, etc. The best classification of geographical origin was obtained by DA, where the samples were clearly divided into 3 groups (Northern and Southern of Italy and Sicily). FA and PCA divided samples into 2 groups with 5 outliers. According to the factor loadings, the most important parameters influencing the FA were TPC (which was most given by quantities of HTY-EA and TY-EA) and the amount of EA, that is the parameter of olives ripeness. Two outliers were samples containing very high TPC. The other three outliers were probably prepared from over-ripened olives because the amount of EA was significantly higher in comparison with the other samples.

HPLC determination of PPs is complicated by many critical issues: standards unavailability; presence of many chemical forms; necessity of extraction method; and PPs high instability in relatively mild conditions. Therefore, the second approach of this work was the comparison of two complementary analytical methods, HPLC and NMR. For this purpose, 32 EVOOs were simultaneously analysed by NMR without any sample pre-treatment or previous separation, and afterwards, three compounds quantifiable through both techniques were compared. Consistent HPLC-DAD and NMR profile was obtained for TY-EA and HTY-EA derivates. Quantification of HTY-EDA provided different values depending on the technique used and NMR showed to be more accurate in this case. Therefore, the innovative and original combination of these approaches turned out to be a very good starting point for a cross-check analysis. This study can pave the way for new projects harnessing the combination of the targeted NMR and HPLC-DAD determinations. Future research can enlighten more

details about this topic by exploiting this complementary approach over a bigger set of samples or different food matrices.

2 Amino acids in drinks

2.1 Introduction

Mead is one of the oldest alcoholic beverages [55,56]. Traditional mead production includes few crucial steps. First, honey is diluted with water and pH value is adjusted and kept throughout the fermentation within a range of 3.6–4.0 [57–61]. The next step is elimination of proteins and undesirable microorganisms by warming or mild cooking [62] followed by tempering to 20–35 °C, inoculation and fermentation by osmotolerant yeast strains [59,63–65]. After decantation, filtration and centrifugation, the mead is filled to the bottles, pasteurised and maturated [59,66]. It has been found that the application of high temperature prior to the fermentation ("hot process") leads to the degradation of valuable thermolabile substances, browning, oxidation and increasing of 5-hydroxymethylfurfural content. Therefore, new but technologically more demanding methods of mead production without any heat treatment ("cold process") have already been developed [62,67,68].

The most common methods of mead adulteration are partial or complete replacement of honey by cheaper molasses or other sugar solutions or mixing honey with ethanol [69]. Therefore, a comprehensive research is necessary to be carried out to prevent mead adulteration and protect the consumers and righteous producers against this act [70–72].

Honey is source of many biologically active compounds such as PPs, amino acids, enzymes, and minerals, which can be transferred to the mead during its production. Despite all the substances, our attention was given to amino acids (AAs) which contribute to the final aroma, flavour and colour of mead and can be used for the mead authenticity assessment [73–76].

HPLC is the most common method of AAs determination. Due to the absence of chromophore or fluorophore as well as low ionization efficiency of AAs, pre-column derivatization step is usually necessary for its spectrophotometric, fluorescence or mass spectrometric detection [74,75,77].

This study was focused on development of simple and rapid AAs analysis using HPLC-UV/VIS after their derivatization by phenyl isothiocyanate (PITC) and dansyl chloride (DNS-Cl) agents. The optimised method was applied on the samples of meads and energy drinks [78,79].

2.2 Results and discussion

2.2.1 Preparation and derivatization of samples

Mead is a very complex matrix with high content of sugars and other compounds. Therefore, sample treatment together with the derivatization process must be carefully optimised.

AAs are frequently isolated from matrices by solid-phase extraction (SPE) on cation-exchange SF [75,80–83] using NaOH elution solvent. The extraction process was optimised in the terms of kind of SPE columns, type, concentration and amount of eluent, and initial amount of mead and its pH. However, most of the derivatization agents provide optimal, selective, quantitative, and reproducible reaction with minimal number of by-products in the range of pH 9–10. Consequently, the adjusting of the eluate pH using different acids was also carried out. The best proline (Pro) isolation with recovery 84.2 % was obtained using Strata SCX column combined with 1M NaOH as eluent. Unfortunately, the optimised extraction method of AAs could be employed only prior DNS-Cl derivatization [78] which is not as sensitive of high pH as PITC derivatization.

Originally, the DNS-Cl derivatization process was based on a relatively complicated procedure [84,85], where AAs react with DNS-Cl in the presence of lithium carbonate. The reaction mixture was heated at 40 °C for 20 min, and derivatives were analysed after 30 min of 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 on the appropriate amount of derivatization agent required for the reaction, real reaction time, the need to include a heating step and equilibration time, and the function of lithium carbonate in the reaction. The optimised derivatization procedure was very simple, the mead extract was only mixed with lithium carbonate and DNS-Cl solution. After 5 min at room temperature and in the darkness, the derivatization mixture was analysed. Obtained derivatives remained unchanged for at least 24 hours [78].

The PITC derivatization procedure of AAs was optimised with respect to the amount and type of used solvents, total derivatization time, procedure suitability for mead samples and quality of acquired chromatograms. Two previously presented derivatization approaches [80,86] were tested. Derivatization procedure according to Komarova et al. [86] was based on the extraction of obtained derivatives by *n*-hexane. This derivatization procedure showed very good repeatability, only one drying step was included, and simple reagents mixtures were used. However, the process was significantly prolonged by two-step extraction. Therefore, our attention was paid to the derivatization procedure developed by Davey, & Ersser [80]. The drying of the sample had to be included prior the derivatization and after each partial reaction. Due to these continual drying procedures, the total derivatization time was very prolonged which also significantly influenced the reaction repeatability. Therefore, the new PITC derivatization method without any extraction and drying steps throughout all the process was developed. Mead sample was adjusted to pH 5 and mixed with reaction solution composed of sodium acetate, triethylamine, and PITC. After 20 min of the

stirring, the suspension was centrifuged and the liquid layer was separated, filtered, and analysed. The developed method is rapid, instrumentation and work unpretentious, provides good repeatability and no impurities or reaction by-products are visible in the chromatograms. Regarding the stability of obtained PITC derivatives, these remain unchanged for several weeks at 5 °C.

Later, the same derivatization procedure without any prior extraction was applied also for the determination of taurine in common energy drinks.

2.2.2 Optimisation of RP-HPLC analysis

DNS-Cl derivatives

Parameters such as type of an organic component of the MF (ACN, MeOH, and their mixture), pH of aqueous MF component (2.5–3.5), influence of TEA addition (0–0.2 %) to the aqueous MF component as well as various gradient slope with different initial and final concentration were tested during optimisation of DNS-Pro analysis. It was found that the best separation with symmetric and narrow peaks was obtained with the gradient elution with binary MF comprised of 0.1% TEA in water acidified to pH 3.0 and 100% ACN, the gradient elution program was following: 40–100 % B in 4 minutes. The excitation and emission spectra of the prepared derivative were measured on the spectrofluorimeter to find the suitable wavelengths for the HPLC fluorimetric detector. DNS-Pro derivative shows excitation and emission maximums at 264 nm and 497 nm, respectively.

PITC derivatives

The optimisation of HPLC separation of PITC-AAs derivatives was very challenging process because the separation selectivity of AAs derivatives and thus quality of separation is affected by many parameters such as SF, type and pH of MF, and temperature as well as composition of injected sample. Two different columns (Ascentis Express C18 and Zorbax SB-Aq) were tested but due to the high polarity of PITC derivatives, very high concentration of water in MF was necessary for their separation. Therefore, the Zorbax SB-Aq column was much more suitable for the derivatives separation in such an aqueous environment. The influence of various pH (5.2–6.55) of the aqueous MF component (70mM ammonium acetate) was investigated. It was found that even a minimal change of the pH value causes significant changes in the separation selectivity. Further, a composition of organic MF part plays also very significant role. Mixtures of ACN/H_2O, MeOH/H_2O, and their combinations (ACN/MeOH/H_2O) were tested. The best separation efficiency and good selectivity of the separated derivatives were achieved using 70mM ammonium acetate with pH 6.55 combined with ACN/H_2O. Finally, 20 PITC-AA derivatives out of 21 analyzed were successfully separated in rapid 10 minutes run with unit resolution or better. Only one co-elution was observed in case of isomeric leucine and isoleucine, which have very similar properties and identical dissociation constants. Final optimised chromatographic separation with the peak description is shown in the Fig. 7.

The identical protocol with just slight modification of gradient program was used also for analysis of taurine in energy drinks. In this case, the gradient elution of mobile phases was very fast and took only 3 minutes.

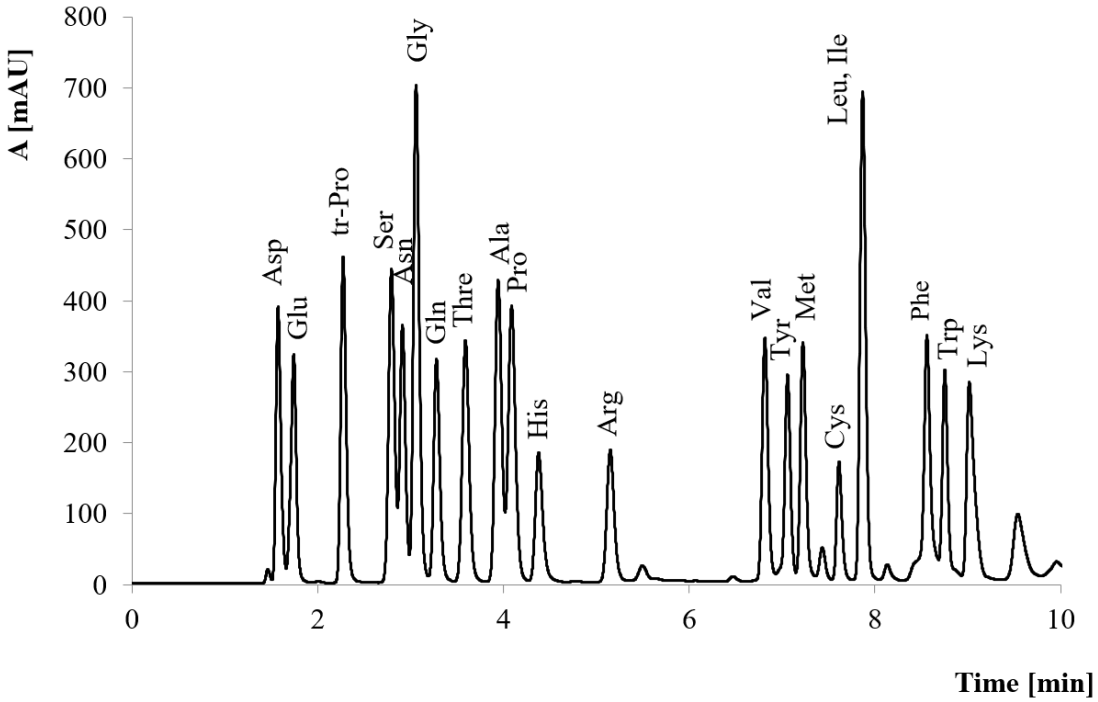

Fig. 7 Optimised separation of 21 AAs standards after PITC derivatization.
Zorbax SB-Aq (150 x 3 mm; 3.5 μm), MF A: 70mM ammonium acetate; pH 6.55, MF B: 60% ACN, gradient: 0 min – 6 % B, 1 min – 10 % B, 4 min – 18 % B, 8.5 min – 60 % B, 9 min – 100 % B, 0.6 mL/min, 254 nm.
Notes: Asp = Aspartic acid, Glu = Glutamic acid, tr-Pro = *trans*-4-hydroxy-L-Proline, Ser = Serine, Asn = Asparagine, Gly = Glycine, Gln = Glutamine, Thre = Threonine, Ala = Alanine, Pro = Proline, His = Histidine, Arg = Arginine, Val = Valine, Tyr = Tyrosine, Met = Methionine, Cys = Cysteine, Leu = Leucine, Ile = Isoleucine, Phe = Phenylalanine, Trp = Tryptophan, Lys = Lysine.

2.2.3 Quantitative analysis of proline in meads using DNS-Cl

Proline is a dominant AA in honey, and thus also mead. Therefore, it can be considered as a marker of mead quality and good manufacturing practise. According to the regulation of the European Parliament and of the Council [87–89], its minimal content must not be lower than 50.4 mg/L in mead. For the evaluation of the quality and authenticity of 24 Czech meads, proline was isolated by SPE extraction, derivatized with DNS-Cl agent, and analysed using HPLC. The quantitative analysis was performed by calibration curve method and final proline contents are shown in the Fig. 8. From this figure it is evident that samples No. 2, 4, and 13 contain the highest

proline amount. All of them were produced by local beekeepers and were prepared by cold way.

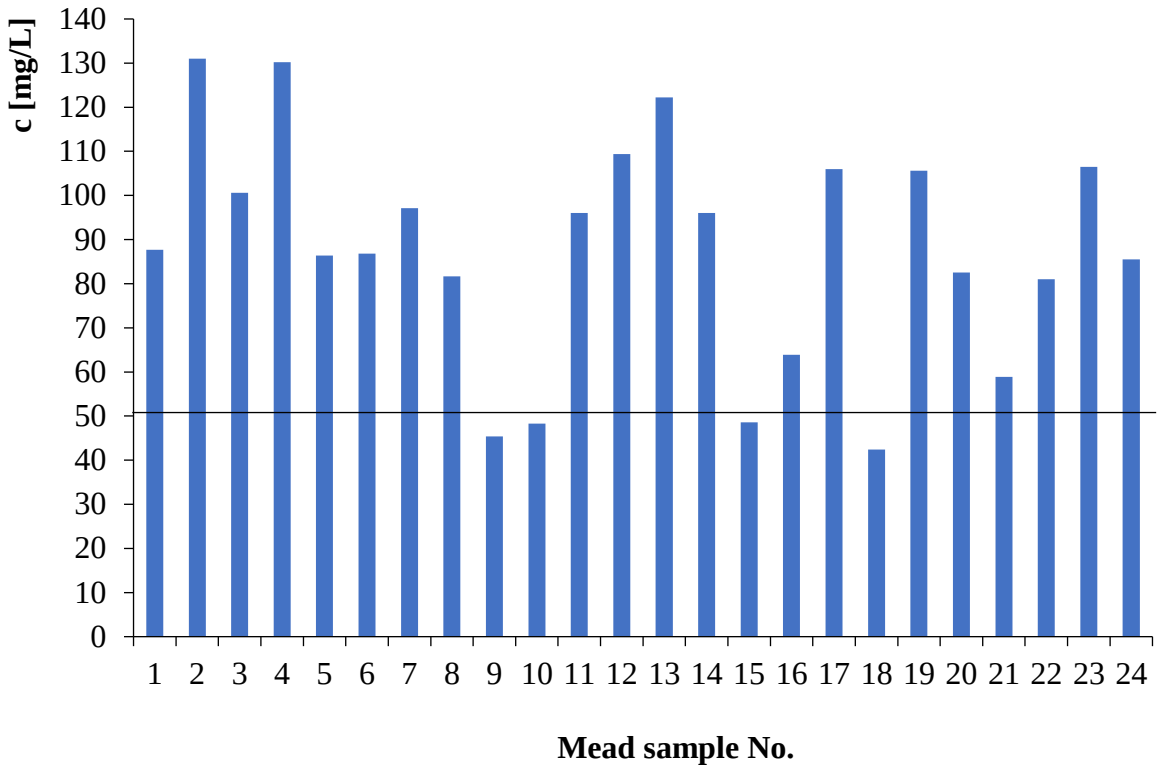

Fig. 8 Proline content in 24 Czech mead samples including minimum quantity line at 50 mg/L.

On the other hand, four samples (No. 9, 10, 15, and 18) did not reach the requirements for the minimal proline content (Fig. 8). All of them were obtained in the markets. Producers of these meads evidently did not comply with the good manufacturing practice related to the requirements of minimal amount of used honey for the mead preparation or they used honey of low quality. Therefore, these samples can be considered as adulterated.

2.2.4 Quantitative analysis of AAs in meads using PITC

The three-step multiple point standard addition method was chosen for the determination of AAs derivatized by PITC in 5 Czech meads. Different qualitative and quantitative AAs profiles were found (Table 1).

Proline, tyrosine, glycine, phenylalanine, leucine, and isoleucine were determined in all samples. On the other hand, threonine, histidine, and tryptophan were not detected. The total AAs amount varied in the range of 134–828 mg/L. Concentrations of proline ranged from 30 to 266 mg/L. The highest proline concentration was determined in meads produced by beekeepers, namely in the samples No. V and IV (266 mg/L and 173 mg/L, respectively), which simultaneously

corresponds to the highest total AAs content (569 mg/L and 828 mg/L, respectively) in these samples. On the contrary, the mead No. I comprised very low total AAs content. Furthermore, this sample contained only 30.0 mg/L of proline, thus, it did not meet its minimal limit (50.4 mg/L) [87–89]. This manufacturer probably did not follow crucial rules of good manufacturing practise and therefore, this sample can be considered as adulterated mead. Due to the absence of already published work focused on determination of AAs in meads, our results had to be compared with Harmonised methods of the International Honey Commission [90]. The results of this method obtained by two approaches of the external standard quantification (calibration curve and direct comparison) were in a good agreement with those determined by developed HPLC method.

Table 1 The concentration [mg/L] of presented AAs in 5 mead samples.

AAs	**SAMPLE NUMBER**				
	I	**II**	**III**	**IV**	**V**
Asp	-	-	-	85.9 ± 1.4	82.8 ± 1.3
Glu	-	24.2 ± 1.3	18.5 ± 0.6	38.7 ± 1.3	28.4 ± 1.7
tr-Pro	4.6 ± 1.0	241.5 ± 2.6	-	4.5 ± 0.8	3.1 ± 0.7
Ser	-	-	-	-	75.4 ± 1.4
Asn	-	44.2 ± 1.5	46.1 ± 1.0	133.6 ± 2.1	39.6 ± 1.0
Gly	14.8 ± 0.9	11.3 ± 0.6	12.3 ± 1.0	19.2 ± 1.0	19.8 ± 0.8
Gln	-	19.7 ± 1.9	21.9 ± 1.7	41.2 ± 1.6	-
Thre	-	-	-	-	-
Ala	29.8 ± 1.2	36.7 ± 1.5	30.0 ± 0.7	81.3 ± 1.2	-
Pro	**30.0 ± 1.6**	**82.6 ± 1.4**	**90.5 ± 1.5**	**172.7 ± 1.5**	**265.7 ± 0.7**
His	-	-	-	-	-
Arg	-	-	-	-	-
Val	1.5 ± 0.4	-	-	31.9 ± 1.6	19.1 ± 0.7
Tyr	12.0 ± 0.6	25.3 ± 1.8	13.6 ± 0.3	15.7 ± 0.8	13.4 ± 1.1
Met	17.3 ± 0.6	19.3 ± 0.5	-	11.2 ± 0.6	-
Cys	-	-	-	112.7 ± 1.7	-
Leu + Ile	6.9 ± 0.4	7.6 ± 0.8	7.1 ± 0.8	14.8 ± 0.8	8.8 ± 0.9
Phe	7.2 ± 0.9	11.5 ± 0.4	12.2 ± 0.3	19.3 ± 0.5	12.3 ± 1.0
Trp	-	-	-	-	-
Lys	9.5 ± 1.6	-	27.6 ± 1.3	44.8 ± 1.9	-
Total AAs amount	**134 ± 0.9**	**524 ± 1.3**	**280 ± 0.9**	**828 ± 1.2**	**569 ± 1.0**

Notes: description of AAs abbreviations is in the Fig 7.

2.2.5 Quantitative analysis of taurine in energy drinks

Taurine is sulphuric [91], non-proteinogenic [92,93], and non-essential β-AA [93] which belongs to fundamental ingredients of energy drinks (usually 0.4% content) [91,94]. Due to the many serious side effects associated with its excessive use, especially when combined with caffeine, alcohol, or guarana (energy drinks are a cocktail of all these substances), it is necessary to develop rapid and accurate determination [92,95]. The taurine quantitative analysis was performed by calibration curve method after its PITC derivatization and RP-HPLC separation. Further, the laboratory protocol of this method was validated in the terms of precision, accuracy, and linearity. Precision of the method was determined (RSD = 2.9 %) using repeated measurements (n = 10). Accuracy describing a difference between determined and true analyte content was characterized using calculation of recovery. A satisfactory recovery value (102.1 %) was achieved. Linearity was very good with the coefficient of determination $R^2 = 0.9982$ [79].

Concentrations of taurine in energy drinks were also determined using square-wave voltammetry (SWV) and the results were compared with those obtained by HPLC. For this purpose, the derivatization of taurine by OPA agent was optimised. At first, the OPA derivatization reaction was characterized (reaction kinetics, ratios of components used etc.) using ultraviolet spectrophotometry from 200 to 400 nm. It was found that the derivatization mixture (OPA and ethanethiol diluted in methanol) and standard solution or diluted sample had to be stirred in the accurate ratio for 6 min to reach reproducible and quantitative reaction. Further, the optimised derivatization method was utilized by SWV at a glassy carbon electrode, in which the direct voltammetric oxidation of the OPA-ethanethiol-taurine derivative was monitored.

Chromatographic as well as voltammetric analysis of samples were five times repeated (n = 5) and final results are calculated and presented as confidence intervals $\bar{x} \pm st_{1-\alpha}$, where $\bar{x}$ is the mean value, s the standard deviation, and $t_{1-\alpha}$ the critical value of Student's t-distribution for five (2.015) or for three (2.353) repetitions at a significance level α of 0.05 (95% probability). If the confidence intervals for HPLC and reference SWV method overlap (Table 2), it should be evident that developed method is comparable.

Table 2 shows values that are presented as arithmetic means with corresponding confidence intervals for repetitions used. About 90 % of declared taurine content was found using both analytical methods. It can be concluded that more precision results were obtained by RP-HPLC method (lower values of confidence intervals). However, developed voltammetric method provides comparable analytical results and due to a lower consumption of organic solvents, lower initial cost of instrumentation, and statistically comparable analytical parameters can be attractive for practical laboratories [79].

Table 2 Comparison of standard HPLC with reference SWV method in analysis of selected energy drinks.

SAMPLE	Concentration of Taurine [mg/100 mL]		
	SWV	HPLC	Declared content
Crazy wolf classic	319.0 ± 29.2	302.6 ± 11.4	350
Crazy wolf with currant flavour	313.0 ± 8.7	311.3 ± 0.4	350
Red Bull classic	351.4 ± 34.9	357.1 ± 6.9	400
Red Bull summer edition	352.0 ± 1.8	349.6 ± 7.0	400
Semtex no sugar	355.0 ± 16.7	334.2 ± 10.6	400
Semtex grapefruit with tonic	328.0 ± 7.7	333.6 ± 6.5	400

2.3 Conclusion

Mead adulteration is particularly topical issue mainly due to the lack of regulations in the mead production. Therefore, it is necessary to establish more analytical methods of the mead characterization revealing the producers who do not follow the correct technological process. In this work, we focused on determination of amino acids from which the proline is the dominant. At first, proline was isolated using SPE extraction on Strata SCX column and obtained extract was derivatized by DNS-Cl agent. Then, RP-HPLC coupled with the fluorescence detector was used for proline determination in 24 Czech meads. The amount of proline ranged from 42.4 ± 3.1 to 131.0 ± 3.3 mg/L in the samples. In general, commercial samples contained lower proline content than those purchased from beekeepers. Four samples did not meet the required minimum concentration of proline (50.4 mg/L) given by the regulations [87,89]. All these samples were bought in local markets and the producers of these meads apparently did not follow the correct technological procedure. In the second part of work, rapid and easy method of determination of AAs profile in meads was developed and optimised. Hence, simple derivatization procedure using PITC agent together with following rapid RP-HPLC separation was developed. AAs derivatives were separated in just 10 minutes. The optimised method was applied on 5 mead samples and the total AAs amount found was in the range of 134–828 mg/L. Content of proline determined by HPLC method was confirmed by harmonised spectrophotometric method of the International Honey Commission and the obtained results were in a good agreement. Among the studied samples, only one of them did not meet the minimum proline level, which indicates that the sample was not produced in agreement with good manufacturing practice.

In the end, the method of chromatographic determination of AAs after their pre-column PITC derivatization was applied for the determination of taurine in the samples of commercial energy drinks. This method was also compared with a simple and rapid electroanalytical method based on direct voltammetric oxidation of OPA-

ethanethiol-taurine derivative at glassy carbon electrode. Obtained results show that the voltammetric method provides statistically identical values and therefore, it could find its application in the routine food analysis for small laboratories which cannot afford to acquire chromatographic instrumentation.

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4 List of Students´ Published Works related to presented dissertation

ARTICLES

Milan Sýs, **Jitka Klikarová**, Lenka Česlová, and Karel Vytřas (2017). Catalytic Activity of Agaricus Bisporus Mushroom Tyrosinase to Acetaminophen in the Presence of L-Proline. *Scientific Papers of the University of Pardubice*, Series A, 23, 85–97.

Amir Shaaban Farag, **Jitka Klikarová**, Lenka Česlová, Karel Vytřas, and Milan Sýs (2019). Voltammetric Determination of Taurine in Energy Drinks after o-Phthalaldehyde-Ethanethiol Derivatization. *Talanta*, 202, 486–493.

Jitka Klikarová, Archimede Rotondo, Francesco Cacciola, Lenka Česlová, Paola Dugo, Luigi Mondello, and Francesca Rigano (2019). The Phenolic Fraction of Italian Extra Virgin Olive Oils: Elucidation Through Combined Liquid Chromatography and NMR Approaches. *Food Analytical Methods*, 12(8), 1759–1770.

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Jitka Klikarová, Lenka Česlová, Petra Kalendová, Paola Dugo, Luigi Mondello, and Francesco Cacciola (2020). Evaluation of Italian Extra Virgin Olive Oils Based on the Phenolic Compounds Composition Using Multivariate Statistical Methods. European *Food Research and Technology*, 246, 1241–1249.

Jitka Klikarová, Klára Jakubů, Lenka Česlová, and Jan Fischer (2020). Determination of Oleocanthal in Extra Virgin Olive Oils Using High-Performance Liquid Chromatography. *Scientific Papers of the University of Pardubice*, Series A, 26. **(ACCEPTED ARTICLE)**

Jitka Klikarová, Lenka Česlová, and Jan Fischer. Rapid analysis of Phenylisothiocyanate Derivatives of Amino Acid Presented in Czech Mead. **(SUBMITTED ARTICLE TO TALANTA)**

PRESENTATIONS

Jitka Klikarová, Lenka Česlová, Francesco Cacciola, Petra Šilarová, Paola Dugo, and Luigi Mondello. Stanovení Fenolických Látek v Italských Extra Panenských Olivových Olejích a Statistické Zhodnocení Jejich Geografického Původu. Monitorování cizorodých látek a hodnocení rizik v životním prostředí I., Kojetín, Czech Republic, 10–12.4.2019. Collection: pp. 75–86, ISBN 978-80-7560-234-3.

Amir Nawar, **Jitka Klikarová**, Milan Sýs, and Karel Vytřas. Analytical Method to Evaluate Taurine in Energy Drinks after o-phthalaldehyde-ethanethiol Derivatization Using Square Wave Voltammetry. XXXIX. moderní elektrochemické metody, Jetřichovice, Czech Republic, 20–24.5.2019. Collection: pp. 57–60, ISBN 978-80-905221-7-6.

Jitka Klikarová, Jana Šebková, Lenka Česlová, and Jan Fischer. Stanovení Aminokyselin Obsažených v Medovinách s Využitím Kapalinové Chromatografie. Monitorování cizorodých látek v životním prostředí XIX, Ovčárna pod Pradědem, Czech Republic, 19–21.4.2017. Collection: pp. 75–84, ISBN 978-80-7560-086-8.

Jitka Klikarová, Lenka Česlová, Soňa Řezková, and Jan Fischer. Stanovení Aminokyselin v Medovinách. Monitorování cizorodých látek v životním prostředí XVIII, Ovčárna pod Pradědem, Czech Republic, 30.3.–1.4.2016. Collection: pp. 57–66, ISBN 978-80-7560-005-9.

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Jitka Klikarová, Oriana Colletta, Francessco Cacciola, Paola Dugo, Lenka Česlová, and Luigi Mondello. Optimization and Validation of an HPLC-PDA/ESI-MS Method for the Determination of Polyphenols in Extra Virgin Olive Oils. 42nd International Symposium on Capillary Chromatography and 15th GCxGC Symposium, Riva del Garda, Italy, 13–18.5.2018. Collection: pp. 489, ISBN 978-88-941816-1-6.

Jitka Klikarová, Lenka Česlová, and Jan Fischer. HPLC Separation of Derivatized Amino Acids in Mead. HPLC 2017, Praha, Czech Republic, 18–22.6.2017. Collection: pp. 277, ISBN 0-8247-4710-0.

Jitka Klikarová, Jana Šebková, Lenka Česlová, and Jan Fischer. Optimization of HPLC Separation of Amino Acids Presented in Mead. 11th Balaton Symposium on High Performance Separation Methods, Siófok, Hungary, 6–8.9.2017. Collection: pp. 94, ISBN 978-615-5270-38-3.