Acta Societatis Botanicorum Poloniae

SHORT COMMUNICATION

ATR-FTIR-based fingerprinting of some Cucurbitaceae extracts: a preliminary study

Wirginia Kukula-Koch[1]*, Maciej Grzybek[2], Aneta Strachecka[3], Aleksandra Jaworska[4,5], Agnieszka Ludwiczuk[1]

[1] Chair and Department of Pharmacognosy with Medicinal Plants Unit, Medical University of Lublin, Chodźki 1, 20-093 Lublin, Poland
[2] Department of Tropical Parasitology, Medical University of Gdańsk, Powstania Styczniowego 9B, 81-519 Gdynia, Poland
[3] Department of Biological Basis of Animal Production, Faculty of Biology and Animal Breeding, University of Life Sciences in Lublin, Akademicka 12, 20-950 Lublin, Poland
[4] Faculty of Chemistry, Jagiellonian University, Ingardena 3, 30-060 Cracow, Poland
[5] Jagiellonian Center for Experimental Therapeutics, Jagiellonian University, Bobrzyńskiego 14, 30-348 Cracow, Poland

* Corresponding author. Email: virginia.kukula@gmail.com

Abstract

The attenuated total reflectance-Fourier transform infrared (ATR-FTIR) fingerprinting of some selected cucurbits was performed on three types of seed extracts (alcoholic, cold-water, and hot-water) in order to elaborate a characteristic FTIR profile of their family representatives and to determine their biochemical content. Cluster analysis and principal component analysis were performed on *Cucumis melo* subsp. *melo* var. *inodorus*, *Cucurbita pepo*, *C. maxima*, *C. pepo* var. *cylindrica*, *C. maxima* subsp. *maxima* convar. *maxima* 'Hokkaido' to determine the similarities between their seed extracts. The ethanol extract of the *C. pepo* seeds was different from the other seed extracts because in addition to esters, it contained free fatty acids, which could influence its pharmacological activity. The main variable differentiating the extracts was the absorption band at 2,920–2,925 cm^{-1}, which represented saturated fatty acids. The obtained results were analyzed by various statistical tools to evaluate the fingerprints of the selected species of cucurbits.

Keywords

Cucurbita pepo; Cucurbitaceae; pumpkins; FTIR spectroscopy; chemotaxonomy; fingerprinting

Introduction

The Cucurbitaceae plant family consists of 130 genera and approximately 800 species. The plants of this family are collectively known as cucurbits [1]. Pumpkin seeds (e.g., *Cucurbita moschata* and *C. maxima* seeds) have been used as antiparasitic agents in ethnomedicine and reported to have anthelmintic properties when used in humans and livestock [2]. The gastrointestinal (GI) parasites constitute a significant threat for humans, domestic livestock, and wild animals. They are known to be highly prevalent in human populations worldwide, with approximately 3.5 billion people being infected annually [3]. A rising concern is the increasing parasite resistance to synthetic anthelmintics in both humans and animals [4,5]. In view of these dangers, one of the significant steps in overcoming GI infections is to evaluate plants, including the above-mentioned cucurbits, from different geographic locations for their antiparasitic potential [6,7].

Even though the seeds of pumpkins have been extensively used as anthelmintic agents in traditional medicine, the active ingredients of their extracts have not been clearly described so far.

In this study, the Fourier transform infrared (FTIR) spectroscopy technique was used to study the composition of 15 seed extracts from five cucurbit plants in order to

characterize a fingerprint of the representatives of the Cucurbitaceae family and, in light of some recent findings, to confirm the marked activity of the *Cucurbita pepo* extracts in the eradication of the *Heligmosomoides bakeri* nematode in a mouse model [8].

For this purpose, the hot-water, cold-water, and ethanol extracts of *Cucumis melo* subsp. *melo* var. *inodorus*, *Cucurbita pepo*, *C. maxima*, *C. pepo* var. *cylindrica*, *C. maxima* subsp. *maxima* convar. *maxima* 'Hokkaido' were evaluated to determine the eventual variations in their compositions, affected by the usage of various polarity solvents. Furthermore, the obtained results were coupled with the multivariate statistical analyses to evaluate the fingerprint characteristics of these taxonomically related cucurbit species, including several pumpkin varieties, used in veterinary medicine.

Material and methods

Preparation of plant material

The seeds of the above-mentioned cucurbits were purchased from a seed shop in Lublin, Poland, and were deposited in the Department of Parasitology and Invasive Diseases of the University of Life Sciences in Lublin, Poland. They were dried at room temperature, ground with husks using a laboratory blender (700S, Waring, USA), and then stored in the dark at 22°C prior to use. For each species, the hot-water (HWE; at 40°C), cold-water (CWE; at room temperature), and 70% ethanol (ETE) extracts were prepared in triplicate from separate seed batches, using the 10-g portions of the initially moistened seeds (30 min). The seeds were macerated for 4 h in 50 mL of an extractant, using a thermostated magnetic stirrer (ATM Type MM5, Poland). The extract was filtered using a filter paper (grade 390) and then lyophilized (FreeZone 2.5, Labconco, USA). All extracts were prepared and stored at 4°C with limited light access.

ATR-FTIR spectroscopy data processing

The FTIR spectra of extracts were collected by a single-beam Bruker Tensor 27 FTIR apparatus with a deuterated triglycine sulfate (DTGS) detector, a midinfrared (IR) source, and a single-reflection attenuated total reflectance (ATR) diamond crystal. All spectra were recorded in the range of 400–4,000 cm^{-1}, with a spectral resolution of 4 cm^{-1} and 64 scans. Before each analysis, the measurement of the background spectrum was performed. Each spectrum was collected by placing one spoonful of the dried extract on the round crystal window. The OPUS FT-IR Data Collection Program (ver. 1.1) was applied to record and manage the data (smoothed with Savitzky–Golay algorithm).

Statistical analysis

The multivariate statistical analysis, including the cluster analysis (CA) and principal component analysis (PCA), was performed for all 15 extracts obtained from the five cucurbit species, using the Statistica version 10 software (Statsoft, Poland). The cluster analysis was performed using the Ward's method of hierarchical clustering based on the squared Euclidean distance between the pairs of extract samples.

The applied multivariate statistical analysis and principal component analysis (PCA) gained popularity in recent years and are widely used in the differentiation of the complex samples of different origins. The merging of mathematical models with the FTIR spectroscopy has been thoroughly described, especially to provide the data on the quality assessment, fingerprinting, determination of sample adulterations, and authentication analyses [9]. These comprehensive methods found their application, especially in food control, where a quick glance at the association of fingerprints with some model samples is useful while determining the identity of various cultivars [9]. It is worth noting that this approach does not require any intensive chromatographic procedures, thus lowering the costs and durations of analyses.

In the present study, a comparison of the three extracts (hot-water, cold-water, and ethanol extracts) of each of the five studied cucurbits was performed, resulting in the elaboration of a general spectral profile of the representatives of the Cucurbitaceae family, as shown in Fig. 1. Fig. 2 summarizes the particular bands characteristic of all extracts, as well as the data on their intensities in the samples. The spectral analysis pointed out that these plants deliver a set of IR absorption bands in the whole analyzed spectral region ranging from 4,000 to 400 cm^{-1}.

Each sample was found to contain marked quantities of lipids, mainly esters of fatty acids. Distinct bands assigned to lipids, located at approximately 1,735–1,740 cm^{-1} (G; see Fig. 2), representing the stretching of the C=O group, were visible in all tested samples. Furthermore, a stretching of the COO⁻ group located in the region of 1,380–1,404 cm^{-1} (K), of C–O at 1,041–1,072 cm^{-1} (N), and an asymmetric CH_2/CH_3 scissoring at 1,442–1,458 cm^{-1} (J) were detected.

The above-described bands might explain the presence of triglycerides, sterol esters, or phospholipids in the samples. Analyzing the particular extracts, water was found to be a better extractant of the C=O groups present in the components of the extract in most of the cases.

The bands localized at 2,854–2,862 cm^{-1} (E) and 2,920–2,925 cm^{-1} (D) represent the symmetric and asymmetric stretchings of a CH_2 group, whereas the one localized at the 2,985–3,016 cm^{-1} (C) represents the scissoring vibrations of the CH_2 group – all belonging to the unsaturated fatty acids, triglycerides, or unsaturated lipids present in the samples, as confirmed by a band for the N–H vibrations at 3,278–3,294 cm^{-1} (B).
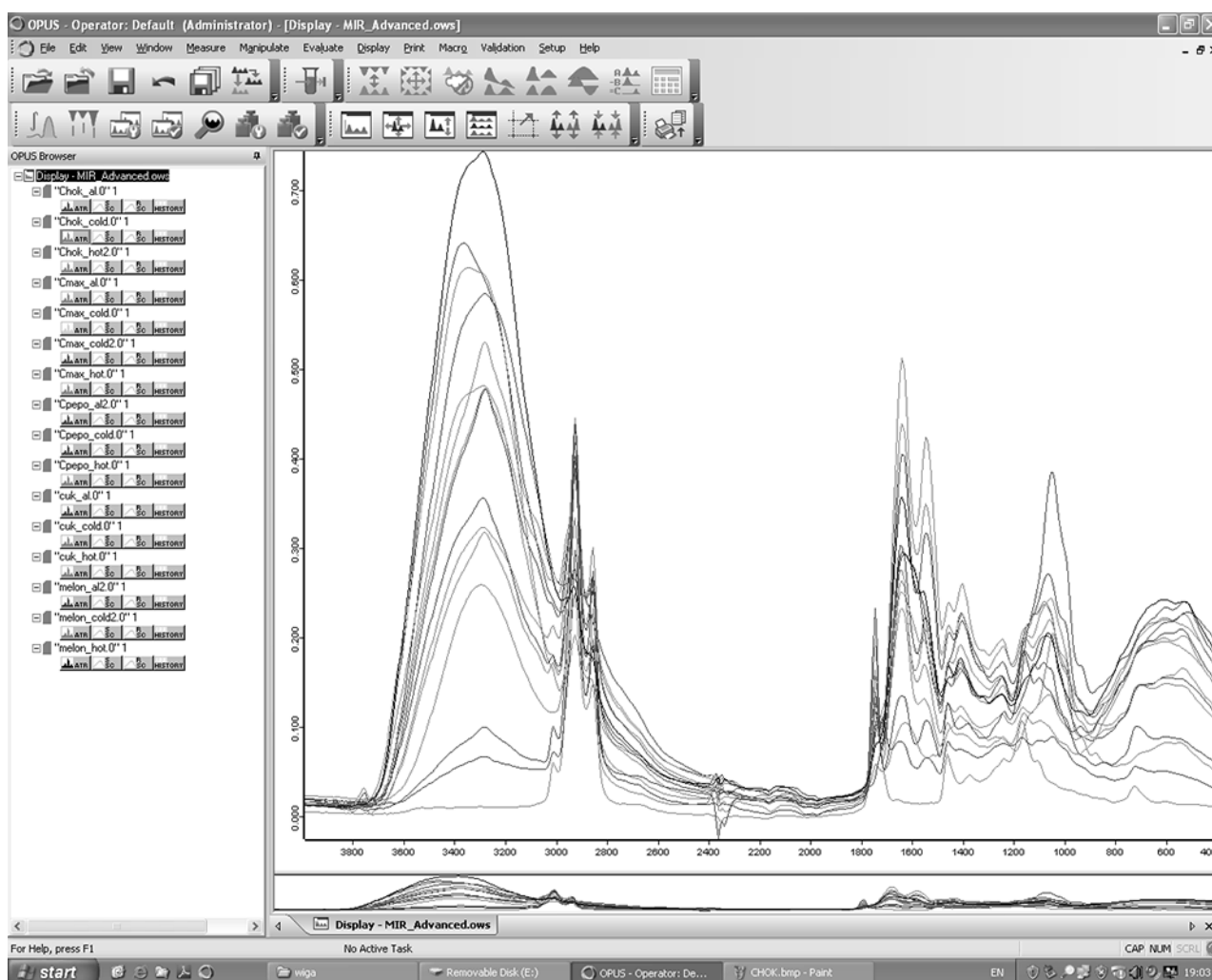

Fig. 1 ATR-FTIR fingerprints of 15 extracts from cucurbits (al – ethanol extract; cold – cold-water extract; hot – hot-water extract; Cpepo – *Cucurbita pepo*; Cmax – *Cucurbita maxima*; melon – *Cucumis melo* subsp. *melo* var. *inodorus*; Chok – *Cucurbita maxima* subsp. *maxima* convar. *maxima* 'Hokkaido', and cuk – *Cucurbita pepo* var. *cylindrica*).
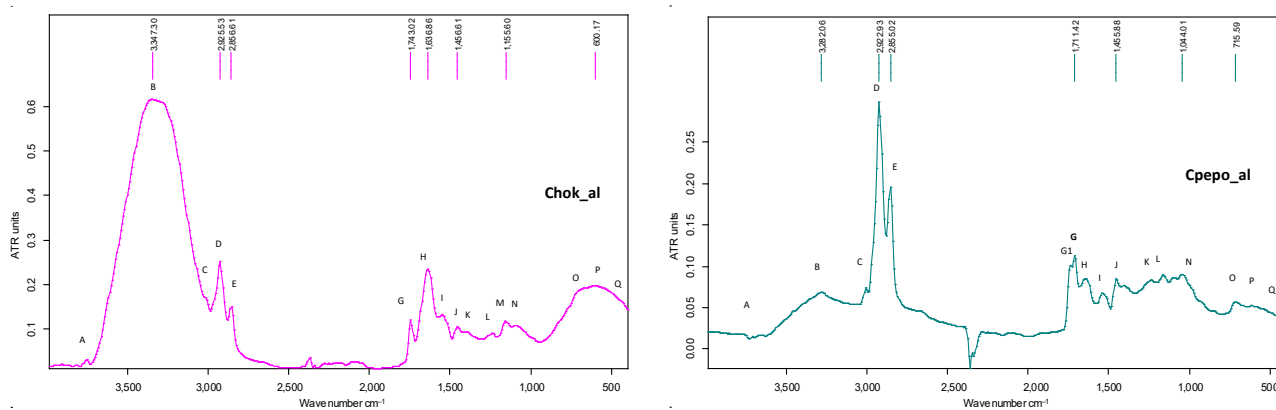
Fig. 2 Sample ATR-FTIR fingerprints of *Cucurbita maxima* subsp. *maxima* convar. *maxima* 'Hokkaido' (left) and *Cucurbita pepo* (right) ethanol seed extracts with their respective stretching assignments.

Three bands confirming the presence of the amides of protein origin were found in the extracts: Amide I at 1,627–1,643 cm^{-1} (H), Amide II at 1,542–1,558 cm^{-1} (I), and Amide III at 1,234–1,242 cm^{-1} (L), connected with the previously described B and C bands, which were also characteristic of proteins.

A vivid exception was noted in the composition of the Cpepo_al extract, in which the vibrations of the C=O group of acids were localized at 1,711 cm^{-1} (see G1 in Fig. 2), likely confirming the relatively high conversion of fatty acids to esters. We speculated that this particular difference might have influenced the activity of the *C. pepo* extracts, which are most commonly known for their antiparasitic properties. Various authors assign the anthelmintic properties of pumpkin seeds to cucurbitin (a nonprotein amino acid), cucurbitacins (terpenoids), or fatty acids [10,11]. However, only a few in vitro or in vivo studies have been performed to identify the pure components of the extracts; as a result, these assumptions have not yet been confirmed. Pineda-Alegria and coinvestigators suggested anthelmintic properties of fatty acids [12], showing, as an example, a fraction composed of five compounds, including four were fatty acids (pentadecanoic, hexadecanoic, octadecanoic, and octadienoic acids) and one sterol (β-sitosterol). In this example, the antinematicidal role of an acidic group could be confirmed [12]. In view of these observations, the presence of acidic bands in the FTIR spectra of cucurbits might influence their pharmacological significance.

The statistical analysis of the FTIR spectra helped to arrange the obtained extracts in accordance with their similarity. In Fig. 3, the hierarchical dendrogram of the linkage of the examined extracts is presented. The graph clearly shows the division of all extracts into two groups – the first group consisting of water extracts and zucchini alcoholic extract (Cuk), and the second one, similar to the Cpepo_al extract, comprises the majority of alcoholic extracts and a cold-water extract from Chok_cold and is highly different from the former. Fig. 3 confirms the selectivity of the extractants applied and shows variable chemical composition of the aqueous and ethanol extracts of the cucurbits.

The existence of chemical differences between the alcoholic and water extracts is also evident from the PCA analysis. The FTIR data showed a quite good separation
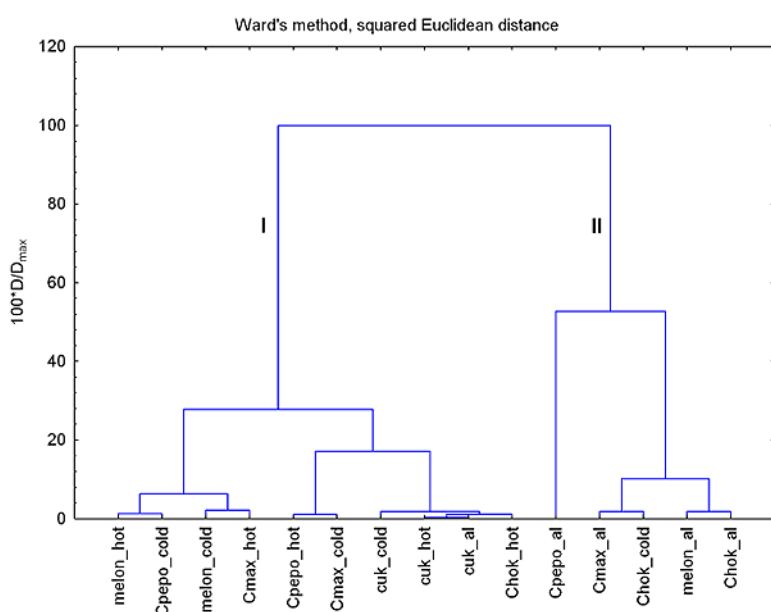

Fig. 3 Hierarchical dendrogram representing the chemical similarities (based on the FTIR spectra) between the analyzed extracts (for abbreviations, see Fig. 1).
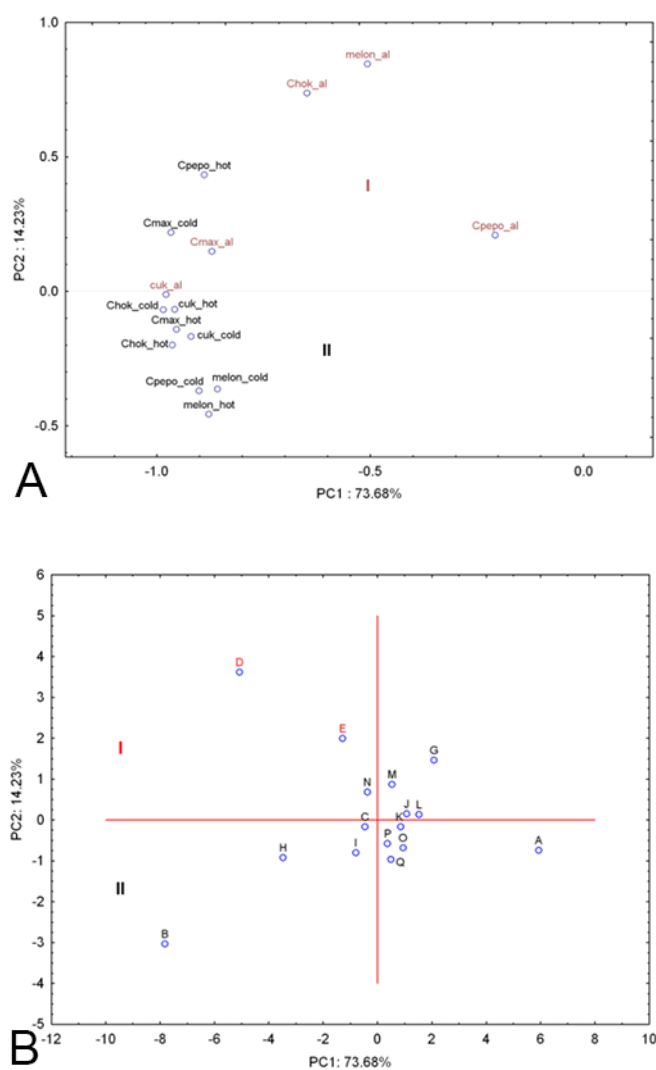
Fig. 4 Principal component analysis (PCA) of the FTIR vibrations characteristic of the examined extracts. (**A**) Factor scores plot (PC1 vs. PC2). For abbreviations of extracts, see Fig. 1. (**B**) Factor loadings plot (PC1 vs. PC2). For band characteristics, see Fig. 2.

between both kinds of extracts (Fig. 4A). All ethanol extracts (Group I) obtained from the Cucurbitaceae seeds were located on the positive side of the PC2 axis, while the water extracts (Group II) appeared on the negative side. The loadings plot, illustrated in Fig. 4B, shows that Group I is differentiated mainly by the Vibrations D and E, characteristic of fatty acids esters, while Group II is differentiated by the Vibrations B and H, characteristic of proteins. The results of the multivariate statistical analyses (CA and PCA) of the FTIR spectra, presented in the Fig. 3 and Fig. 4, clearly indicate the difference between the Cpepo_al and other alcoholic extracts. The FTIR spectrum of this extract showed the presence of the vibrations belonging to free acids (G1), besides the bands characteristic of fatty acids esters (G).

The results of the ATR-FTIR analysis of the Cucurbitaceae extracts, confirmed by the multivariate statistical analysis, showed the differences in the chemical compositions of the alcoholic and water extracts. The water extracts contained mainly proteins, while the most characteristic groups of the compounds present in the ethanol extracts were fatty acids esters. Among the alcoholic extracts, Cpepo_al was found to be a different one, because it contained free fatty acids, besides esters (Signal G1). These compounds have been studied previously as potential anthelmintics by Hirazawa et al. [13], who confirmed the significant enfeebling effects of the short- and medium-chained fatty acids against the *Heterobothrium okamotoi* species in in vitro trials.

The detailed analysis of the water and ethanol extracts of the selected cucurbits by ATR-FTIR spectroscopy [14] showed significant amounts of proteins, fatty acids, and esters in their composition. However, statistical tests of the obtained data revealed that among the 15 studied extracts, none exhibited a similar composition to that of Cpepo_al, which is known for its traditional anthelmintic applications. The main variable differentiating these extracts was the absorption band localized at 2,920–2,925 cm^{-1}, which represented saturated fatty acids.

Similar examples of multivariate statistical designs were published by other authors. In the manuscript elaborated by Moreiro and coinvestigators [15], the fingerprinting of genetically modified coffee seeds was presented. It was shown that the FTIR technique could provide sufficient information to differentiate both the cultivars and the solvents used for extraction, while maintaining the signals characteristic of coffee beans. Another study published by Kharbach et al. [16] presented a method evaluated to confirm the quality of Moroccan argan oil, which is often adulterated. According to the authors, the mathematical models applied to the FTIR spectra provided good prediction and helped to distinguish five classes of argan oil, even if they were produced in different regions. Furthermore, this approach was used for the quality and falsification control of the olive oil samples [17]. Similar to the results presented herein, the determination of the fatty acid composition by FTIR spectroscopy was possible in the case of olive oil and resulted in the successful fingerprinting of its composition, as well as its detection and quantification in oil blends.

The above examples explain some possible applications of the FTIR spectroscopy coupled with the PCA and multivariate analyses; these applications can be also related to the studies on the commercially available pumpkins and their seeds. The above-presented characteristic signals can certainly help to confirm the identity of pumpkin seeds in foods or dietary supplements.

References

1. Dhiman K, Gupta A, Sharma DK, Gill NS, Goyal AA. Review on the medicinally important plants of the family Cucurbitaceae. Asian Journal of Clinical Nutrition. 2012;4:16–26. https://doi.org/10.3923/ajcn.2012.16.26

2. Guarrera PM. Traditional antihelmintic, antiparasitic and repellent uses of plants in Central Italy. J Ethnopharmacol. 2009;68:183–192. https://doi.org/10.1016/S0378-8741(99)00089-6

3. Stepek G, Buttle DJ, Duce IR, Behnke JM. Human gastrointestinal nematode infections: are new control methods required? Int J Exp Pathol. 2006;87:325–341. https://doi.org/10.1111/j.1365-2613.2006.00495.x

4. Taylor MA. A larval development test for the detection of anthelmintic resistance in nematodes of sheep. Res Vet Sci. 1990;49:198–202.

5. Sangster NC. Anthelmintic resistance: past, present and future. Int J Parasitol. 1999;29:115–124. https://doi.org/10.1016/S0020-7519(98)00188-X

6. World Health Organization. Research priorities for helminth infections. Technical report of the TDR disease reference group on helminth infections. Geneva: World Health Organization; 2012. (WHO Technical Report Series; vol 972).

7. Assessment report on *Cucurbita pepo* L., semen [Internet]. 2012 [cited 2018 May 28]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Herbal_-_HMPC_assessment_report/2013/03/WC500140759.pdf

8. Grzybek M, Kukula-Koch W, Strachecka A, Jaworska A, Phiri AM, Paleolog J, et al. Evaluation of anthelmintic activity and composition of pumpkin (*Cucurbita pepo* L.) seed extracts – in vitro and in vivo studies. Int J Mol Sci. 2016;17:1456. https://doi.org/10.3390/ijms17091456

9. Rodriguez-Saona LE, Allendorf ME. Use of FTIR for rapid authentication and detection of adulteration of food. Annu Rev Food Sci Technol. 2011;2:467–483. https://doi.org/10.1146/annurev-food-022510-133750

10. Paik S, Kwak HS, Park TH. A facile synthesis of(-)-cucurbitin. Bull Korean Chem Soc. 2000;21:131–132.

11. Elhadi IM, Koko SW, Dahab MM, El Imam JM, El Mageed MAE. Antigiardial activity of some *Cucurbita* species and *Lagenaria siceraria*. Journal of Forest Products and Industries. 2013;2:43–47.

12. Pineda-Alegría JA, Sánchez-Vázquez JE, González-Cortazar M, Zamilpa A, López-Arellano ME, Cuevas-Padilla EJ, et al. The edible mushroom *Pleurotus djamor* produces metabolites with lethal activity against the parasitic nematode *Haemonchus contortus*. J Med Food. 2017;20:1184–1192. https://doi.org/10.1089/jmf.2017.0031

13. Hirazawa N, Oshima SI, Mitsuboshi T, Hata K. The anthelmintic effect of medium-chain fatty acids against the monogenean *Heterobothrium okamotoi* in the tiger puffer *Takifugu rubripes*: evaluation of doses of caprylic acid at different water temperatures. Aquaculture. 2001;195:211–223. https://doi.org/10.1016/S0044-8486(00)00565-2

14. Rodriguez-Solana R, Daferera DJ, Mitsi C, Trigas P, Polissiou M, Tarantilis PA. Comparative chemotype determination of Lamiaceae plants by means of GC-MS, FT-IR, and dispersive-Raman spectroscopic techniques and GC-FID quantification. Ind Crops Prod. 2014;62:22–33. https://doi.org/10.1016/j.indcrop.2014.08.003

15. Moreira I, Scarminio IS. Chemometric discrimination of genetically modified *Coffea arabica* cultivars using spectroscopic and chromatographic fingerprints. Talanta. 2013;107:416–422. https://doi.org/10.1016/j.talanta.2013.01.053

16. Kharbach M, Kamal R, Bousrabat M, Mansouri MA, Barra I, Alaou K, et al. Characterization and classification of PGI Moroccan argan oils based on their FTIR fingerprints and chemical composition. Chemometr Intell Lab Syst. 2017;162:182–190. https://doi.org/10.1016/j.chemolab.2017.02.003

17. Jiménez-Carvelo AM, Osorio MT, Koidis A, González-Casado A, Cuadros-Rodríguez L. Chemometric classification and quantification of olive oil in blends with any edible vegetable oils using FTIR-ATR and Raman spectroscopy. LWT – Food Science and Technology. 2017;86:174–184. https://doi.org/10.1016/j.lwt.2017.07.050