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Aquaphotomics: an innovative application of near-infrared spectroscopy focusing on water

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SUMMARY

In the field of food science, indirect methods which can be used to determine a certain property of the sample by correlating the measured physico-chemical characteristics are widely applied for the detection of food counterfeiting. Near infrared spectroscopy (NIR), complemented by multivariate statistical analyses, is a quick, non-destructive method that does not require sample preparation in most cases. The interactions that can be observed between aqueous systems or aqueous solutions and electromagnetic radiation (light), i.e., the effects of different perturbations on the structural and functional properties of water, are investigated by a new and dynamically evolving area of science called aquaphotomics. It has been proven by a number of research results that by applying the so-called “water-mirror approach”, the detection limits that can be obtained using the conventional approach can be overcome in aqueous systems, and in certain cases components that are present in concentrations that are a few orders of magnitude lower than usual can be detected. The technique of aquaphotomics has been tested in diverse areas of science such as medicine, microbiology, plant physiology or food analysis. In our series of experiments, the detectability of the counterfeiting of ground paprika samples using 0 to 40% corn flour has been investigated by applying the method of aquaphotomics to their solutions. During the evaluation of the results, the first harmonic range of water (1,300-1,600 nm) was used. Spectral patterns were represented on an aquagram, and then a model for estimating the corn flour content was constructed using the Partial Least Squares Regression (PLSR) method. PLSR results showed a strong correlation between the added amount of corn flour and the amount estimated by NIR (near-infrared) spectroscopy. Samples with a lower corn flour content (0-3%) showed a greater absorbance around 1,450 nm, while samples with a higher corn flour content (15-40%) exhibited a greater absorbance between 1,364 and 1,412 nm. These differences are explained by the spectral absorption of water bound with hydrogen bonds to varying degrees. Research results from recent years show that aquaphotomics is a promising technique in many areas of science, including food science.

INTRODUCTION TO AQUAPHOTOMICS

Aquaphotomics is a new area of science aimed at investigating water as a complex molecular system of many conformations having different functionalities, based on the interaction of water molecules and light [1].

GOAL AND BRIEF HISTORY OF AQUAPHOTOMICS

Based on her own and other researchers' earlier results obtained during near-infrared spectroscopic measurements of aqueous media, the establishment of a new field of science was proposed by Roumiana

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Tsenkova, a professor at Kobe University in Japan, in 2005, investigating specifically water-light interactions and organizing the knowledge acquired, and she called this field aquaphotomics [2, 3]. Water-light interactions are proving to be a huge source of information on the structural and functional properties of aqueous systems. Water is one of the basic building blocks of nature. This discipline deals with the comprehensive study of water as the “molecular and energy mirror” of aqueous systems.

During the development of aquaphotomics, the main goal was to establish a common platform and strategy for a better understanding of the functionality of water through water-light interaction, at all frequencies of the electromagnetic spectrum. Most work to date has been performed using near-infrared (NIR) spectroscopy, typically in the first harmonic range (1,300-1,600 nm) of the O-H valence vibration, where many specific absorption band characteristic of water have been identified as a result of previous work [4–6]. Research in this field shows that aquaphotomics can make a significant contribution to water research and to a better understanding of the molecular system of water [1].

Compared to mid- and far-infrared light, NIR enables a deeper penetration, thus providing a more reliable yet fast and non-destructive measurement that is particularly suited to study even intact biological systems [1, 7]. When using NIR spectroscopy in analytical practice (for example, in pharmaceutical applications), water is one of the main barriers to the direct mass determination of the individual constituents [8] because it obscures other constituents in the absorption spectrum due to its significant light absorption, thereby significantly changing the spectrum of the sample to be tested and causing absorption band shifts. Since water exhibits a significant light absorption in the NIR range and changes in its quantity can be measured easily, moisture determination is one of the main and most common applications of NIR spectroscopy. It is used, for example, in agriculture, the food industry, medicine, the pharmaceutical industry, the polymer and textile industries to study water content, hydrogen bonds and hydration state [9].

With the development of aquaphotomics, the collective material and energy mirror properties of water have been described [1]. The so-called “water mirror approach” is one of the basic spectrum analysis and interpretation methods in aquaphotomics. It utilizes the high degree of sensitivity of the hydrogen bonds in water, where the structure of the water is influenced by all components of the aqueous system and the surrounding energies [1]. Each aqueous system is a dynamic arrangement of a network of water molecules. The strength, number and quality of the secondary bonds between the water molecules are influenced by the hydration of the component(s) in the system and the various perturbations together,

as they affect the entire body of water [10]. Any disturbance of the aqueous system causes a change in the conformations of the water molecules, resulting in changes in the absorption bands characteristic of water in the NIR spectrum.

As a result of physical and chemical changes, the absorption pattern changes in each case. These properties of water allow the indirect measurement of small amounts of dissolved substance(s) [11], even if the constituent in question does not absorb light in the NIR range [12]. A common feature in aquaphotomics research is that the matrix of the systems studied is water. Special data analysis methodology is required to extract the information hidden in the complex and multivariate spectra of such systems [13]. The toolkit of aquaphotomics includes the application of multivariate data analysis methods used in NIR spectroscopy, which have become more and more sophisticated over the years, and at the same time extending them.

Through the study of the light-water interaction using the methodology of aquaphotomics, the knowledge describing the role of water is constantly expanding. In quality management systems, it is a growing requirement that the methods used in different research projects be fast, non-destructive and chemical free. The undeniable advantage of the method is that it can be used effectively in both basic and applied research, enabling non-invasive observations. Typically, a holistic approach is adopted, as opposed to the measurement of individual parameters [13].

With the development of aquaphotomics, a new terminology has evolved to describe the subject matter of the study as accurately as possible on the basis of the newly acquired knowledge. Figure 1 illustrates the process of the methodology which allows for the above detailed issues. Following experimental design and precise instrument setup, the samples to be tested are exposed to an interference. After the experiments are performed (and the spectra are recorded), the raw data are analyzed, followed by the pretreatment and analysis of the spectral data. The water absorption bands (WABS) obtained as a result of the analyses are used to identify the activated water absorption bands. Activated water absorption bands create water absorption spectral patterns (WASPs), which can be displayed on aquagrams. The database of water absorption bands is the *aquaphotome*.

WAMACS

These are the *Water Matrix Absorbance Coordinates*. They are the spectral ranges where the specific absorption bands (WABS) characteristic of water are most likely to be found, resulting from the absorptions of light by the different molecular conformations of water in different energy ranges (water modifications,

water molecular structures). In the first harmonic range of the O-H valence vibration (1,300-1,600 nm), 12 WAMACS have been identified experimentally [1].

ACTIVATED WATER BANDS

The absorption bands of water are activated to varying degrees during different perturbations. Based on the activated bands that are repeated consistently in aquaphotomics research, the individual perturbations can be easily identified and characterized. Therefore, the activated absorption bands of water are identified during the analyses.

WASP

Water Absorbance Spectral Pattern. The WASP is defined by the combination of activated water bands caused by the different perturbations, which describes the state of the entire water molecular system. It provides a large amount of chemical and physical information, capturing the structure and dynamics of the system as a whole [10]. The WASP can be used as a holistic biomarker to explore system functionality.

AQUAPHOTOME

The database of water absorption bands and patterns in the entire electromagnetic spectrum is called the aquaphotome. The aquaphotome is the totality of different water molecular structures that are formed by aqueous or biological systems under different conditions. The complete aquaphotome can be defined as a comprehensive database of all separate WASPs, interpreting their functionality in a given system under given conditions. The large database obtained about characteristic water bands can be linked to specific biological functions, it allows rapid comparisons and identifications, and can be further used for biological, chemical and physical predictions and diagnoses [1, 10].

AQUAGRAMS

The aquagram is a new graphical representation of results developed for the concise and clear presentation of the water absorption spectral pattern [14]. The aquagram shows the normalized and averaged absorbance values of different sample groups at selected wavelengths [14, 15]. The values on the axes defined by the activated water absorption bands can be plotted on a radar or linear chart [16].

Aquagrams display a specified number of axes and WABS, in a perturbation- and system-specific way. In the first harmonic band of the water valence vibration, the axes of the aquagram are (generally) based on the previously discovered 12 water matrix absorption coordinates (WAMACS) [13].

The classic aquagram is relative and depends on

the samples included in the calculation. A more advanced version is the standardized aquagram with confidence intervals [17]. This extension makes it easier to determine whether the differences in water absorption spectral patterns (WASP) characteristic of the water displayed on the aquagrams are statistically significant [13].

AQUAPHOTOMICS METHODOLOGY

EXPERIMENTAL SETUP

The key element of aquaphotomics studies is the recording of high quality spectra, for which precision spectrometers (high signal-to-noise ratio, S/N) are required with a high spectral resolution (0.5-1 nm) [18]. In addition to the rules applicable to conventional approach NIR analyses, additional specific elements should be ensured during the experimental design. These include, for example, multiple recording of the spectra of the individual samples, analysis of the same sample with several successive illuminations, and continuous monitoring of the environmental parameters such as temperature and humidity. In addition, pure water is measured during the test according to certain samples numbers; water spectra are used to correct for possible unwanted effects in the environment or the equipment. Recording of the spectra of the experimental samples is carried out randomly, using the same cuvette, between regular pure water samples, paying close attention to precise thermostating of the samples. Precise setting of the measurement conditions is necessary, because in aquaphotomics research we usually look for such tiny spectral differences due to the molecular structure of the water, that even the slightest differences due to the geometry of the instrument or the experimental setup, or temperature fluctuations can interfere with the analyses.

In aquaphotomics experiments, various controlled perturbations are often used, as these help to reveal hidden information. In practice, the most frequently used deliberate perturbations are temperature [6], successive illuminations and varying degrees of dilution [19]. The NIR spectra of aqueous systems are very complex, and the changes in their absorbance spectra caused by the different perturbations are generally very subtle, but nonetheless constant and consistent. A given perturbation does not always produce the same change in the spectrum. In the NIR range, there are four pronounced peaks in the water spectrum at about 970, 1,190, 1,450 and 1,940 nm. Depending on the aqueous system tested, some regions may be more suitable for the analysis and provide more information [1, 19].

SPECTRAL PRETREATMENT METHODS

The first step in analyzing the recorded spectra is the visual inspection of the raw spectral data, which can provide information on both clear outliers and

the necessary spectrum pretreatment methods. The primary purpose of pretreatment methods is to enhance information relevant to the research and to reduce unwanted effects. Many pretreatment methods can be used during data analysis. Noise reduction methods include, for example, averaging and smoothing [20]. The most commonly used noise reduction technique in the methodology of aquaphotomics is the application of a Savitzky-Golay filter (usually with a second order polynomial function and an optimized wavelength range) [21]. To correct baseline shifts, normalization, Standard Normal Variate (SNV) transformation [22, 23] and Multi Scattering Correction (MSC) [23] are often used. „Detrending” is also a possible method for correcting baseline shifts, but it can also be used effectively to eliminate slope changes. The difference between spectra and spectral derivatives allows for a more efficient detection of the absorption peaks, which in the aquaphotomics methodology means primarily the absorption bands of activated water. The two most commonly used methods of derivation in the NIR technique are the Norris-Williams [24] and the Savitzky-Golay [21] derivation. Derivatives solve two basic problems: overlapping peaks and large baseline changes. Additive effects are corrected by the first derivative, while multiplicative effects are also corrected by the second derivative. In aquaphotomics research, the second derivatives are typically used to explore activated water absorption bands [25]. Extended Multi Scattering Correction (EMSC) is one of the means by which the distortion of the spectrum by a known effect can be reduced [26].

CHEMOMETRIC METHODS

To evaluate the large amount of information encoded by the absorption spectra, chemically driven multivariate data analysis, so-called *chemometric methods* are required. In the methodology of aquaphotomics, the first qualitative evaluation of multivariate spectral analysis is most often carried out by principal component analysis, the primary purpose of which is to obtain a deeper understanding of the main patterns underlying spectral variability.

Principal Component Analysis (PCA) is one of the most useful and probably the most widely used exploratory techniques in spectroscopy [27]. Its purpose is to determine the possible relationships between the samples, i.e., it provides primary information regarding the major directions and variance of the data set. It reduces the initial data matrix and compresses the entire matrix into a smaller number of background variables representing it accurately. In the resulting multidimensional space, it defines directions where the variance of the results is the greatest. The first principal component explains most of the variance [28]. The results can be illustrated with scores and loadings graphs. The scores graph shows the positions of the measured

spectra of the individual samples in the transformed space of the newly constructed variables (principal components), allowing us to get to know the major patterns of the samples in the spectral space relative to one another, while the loadings graph displays the importance of the roles of the original variables (wavelengths) in shaping the above-mentioned patterns. With principal component analysis one can identify activated water absorption bands.

Discriminant analysis (DA) is a supervised method (grouping of the samples is defined during the analysis). The Partial Least Squares Discrimination Analysis (PLS-DA) [29] assists in the identification of water absorption bands with the greatest differences between certain predefined groups. The method is based on the PLS regression algorithm, which searches for latent variables with the maximum covariance of the Y variables [30]. During the application of SIMCA (*Soft Modeling of Class Analogies*) [31, 32], unique PCA models are built individually in several steps for each predefined sample group and the projection of additional sample groups is used to analyze the similarity and distinctness of the groups. Ultimately, a so-called discrimination power vector is yielded by the method, showing which wavelengths play the most important roles in discriminating, thus providing information about activated water absorption bands.

Water absorption bands can be used, among other things, to indirectly determine changes in the concentrations of the substances present in the water [12, 13]. The partial least squares regression (PLSR) method generalizes and combines the properties of principal component analysis and multiple regression. Its purpose is to explore the relationship between the dependent variable(s) and the independent variables [33-35]. From the independent variables, i.e., the spectra, it creates latent variables (LV), which are linear combinations of the original variables. LVs are designed in a way so that they are able to describe the variance of the dependent variable to be estimated as much as possible. When using the PLSR, the coefficients of the regression equation are calculated and the results for the individual variables are estimated, which provides information on which wavelengths and water-specific absorption bands change the most with the change of the dependent variable under investigation.

Aquaphotomics is primarily applicable to water-rich systems. Water-soluble molecules present in low concentrations are hydrated with large numbers of water molecules. Hydration around the dissolved molecules also indirectly affects the hydrogen bonds between water molecules that are not involved in the hydration. In this way, water acts as a kind of amplifier. Instead of directly measuring the constituent to be detected, information regarding its concentration is obtained indirectly by measuring changes in the solvent molecules [36].

In two-dimensional correlation spectroscopy, external disturbance (such as temperature, concentration, etc.) is applied to the system during the spectral measurement, allowing for a deeper exploration as a function of time or the disturbance. It extends the spectral region to another dimension and decomposes the overlapping wavelength bands into their components [37]. 2D correlation spectra (in which the similarities and differences between the individual spectral intensities are amplified) are derived directly from the dynamic spectra. In this way, the tracing of spectral intensity can be achieved. The standard deviation (variance) of the spectral intensities from the expected value, which is collected sequentially in the order of certain physical variables (such as time or temperature), is compared to two independent wave numbers [38].

The final step in the analysis is the illustration of the water absorption spectral patterns on aquagrams.

APPLICATIONS OF THE AQUAPHOTOMICS METHOD

Near-infrared spectroscopy is a quick and non-destructive technique with a wide range of applications, further expanded by the aquaphotomics methodology. Since the development of the aquaphotomics discipline, it has evolved into a multidisciplinary field of science [39]. In the first publications, applications such as the diagnostic possibilities of mastitis in dairy cows were interpreted and described based on water absorption bands [40, 41]. In animal biology research, the method was used to detect the reproduction period of giant pandas [15] and cows [42].

The method has been used in medical research to detect HIV infection [43], to increase dialysis efficacy, for early screening of colon cancer, and to characterize soft contact lenses [44]. In the microbiological applications of the method, the rapid identification of various lactic acid bacteria strains [45], as well as the possible distinction and resistance of probiotic and non-probiotic lactic acid bacteria [46, 47] have been investigated. In plant biology studies, the diagnosis of soybeans infected with mosaic virus has been carried out [48], and the extreme dehydration tolerance of the Rose of Jericho (*Haberlea rhodopensis*) was confirmed based on water molecular structure [49].

In food industrial applications of aquaphotomics, the quality characteristics of milk [40, 41], and the ochratoxin A contamination of green coffee beans were investigated [50], the shelf life of Italian Taleggio cheese was studied [51] and the ripening of Bulgarian yellow cheese (Kashkaval) has been successfully monitored [52]. In their work, Barzaghi, Cremonesi & Cattaneo identified the bioactive ingredients of Italian propolis in intelligent food packaging materials. The purpose of the work of Vanoli et al. was to study the effect of an innovative,

cellulose polymer based coating on the maturation of Salame Piacentino [53, 54].

Water quality research [55] and experiments in aqueous solutions have also contributed to the widespread recognition of aquaphotomics. Studies have been carried out to measure the pesticide content of water [56], as well as to detect the presence of contaminants in saline solutions [19], among other things. The water mirror approach has allowed the indirect determination of components present in the ppm (1:10⁶), and in some cases even the ppb (1:10⁹) range, which was previously not possible using the conventional NIRS analytical technique [6, 7, 11]. Research has shown that, with this concept, the spectral pattern of water can be used as a holistic water quality indicator [25].

In our recent research, the detectability of counterfeiting of ground paprika with corn flour was tested using the aquaphotomics technique. In the course of the investigations, ground Hungarian sweet paprika was counterfeited with corn flour at concentrations between 0 and 40%, in several steps. The samples thus prepared were diluted tenfold with distilled water, and the solutions were filtered after homogenization. Three parallel transmittance spectra of the resulting solutions were recorded using a MetriNIR (MetriRep Kft., Hungary) instrument in the 740-1,700 nm wavelength range. Evaluation of the spectra was carried out in the 1,300-1,600 nm range. Savitky-Golay smoothing and multiple scatter correction (MSC) were applied to the spectra, and then a model was constructed using the partial least squares regression (PLSR) method to estimate the concentration of the added corn flour. To represent the WASP, a classic aquagram was created. Data were evaluated using the R-project and R-studio 3.5.2. software.

The results of the PLS regression model showed a close correlation (model construction: $R^2=0.97$; model validation: $R^2CV=0.90$) between the estimated and actual amounts of added corn flour. During the construction of the model, we were able to correctly estimate the amount of added corn flour with an error of 2.14%, while the error during the validation was 4.04%.

The results of the aquagram (**Figure 2**) showed that the samples added with less amount of corn flour (1%, 3%) or no corn flour at all had higher absorbance values at wavelengths of 1,426 nm, 1,440 nm, 1,452 nm, 1,462 nm, 1,476 nm and 1,488 nm, which indicates that there are more hydrogen bonded water molecules in the authentic sample and the samples containing 1% or 3% corn flour. In contrast, samples containing more corn flour had higher absorbance values in the 1,364 nm, 1,374 nm, 1,384 nm, and 1,412 nm. The latter suggests that samples containing higher amounts of corn flour (15-40%) contain fewer or weaker hydrogen bonded

water molecules [57]. For the above wavelengths, a certain trend can be observed as a function of the increasing concentration of added corn flour: a decreasing trend between 1,426 and 1,488 nm, and an increasing trend between 1,364 and 1,412.

SUMMARY

The aquaphotomics technique is a new and promising method for the rapid and non-destructive exploration of the structural and functional properties of aqueous systems. In classical NIR spectroscopy, due to its significant light absorption, water alters the spectrum of the analytical sample, making it one of the most common constituents that interfere with direct mass determination. Secondary bonds in aqueous systems (such as hydrogen bonds) react with high sensitivity to any perturbation by changing the conformations of the water molecules, resulting in an absorption band characteristic in water in the NIR spectrum. This is what aquaphotomics takes advantage of. As a result of the different perturbations, whether physical or chemical, the absorption pattern is altered, and so using the spectral signal responses of water, it is sometimes possible to detect components that are present in quantities even a few orders of magnitude lower than in the case of conventional NIR analysis.

The quality of ground products made from paprika is determined by health safety, as well as values for use (shelf life, packaging), nutritional physiology and enjoyment. Perhaps the most important quality indicators of commercially available ground paprika are color and taste. Hungarian paprika is a profitable and important export product, which may justify counterfeiting. Food counterfeiting is also a food safety issue. To detect food counterfeiting, whether by natural or artificial substances, indirect methods are commonly used. Rapid, reliable, non-destructive and chemical-free testing methods are especially common in food quality assurance. NIR spectroscopy, complemented by multivariate statistical analysis, is perfectly suitable for this purpose. The aim of the present study was to use the aquaphotomics methodology for the investigation of ground paprika samples counterfeited with corn flour. The methodology includes experimental design, setting up the measurement system, performing the individual experiments in random order under defined perturbations, analysis and pretreatment of raw data and data analysis using chemometric methods. The purpose of these is to identify absorption bands characteristic of water and corresponding to the perturbation (concentration change), and to summarize the spectral pattern thus determined on an aquagram.

The results of the PLS regression model showed a strong correlation (model construction: $R^2=0.97$; model validation $R^2CV=0.90$) between the amounts of corn flour estimated by NIR and the actual amounts added. The model was able to correctly estimate the

amount of corn flour added with an error of 2.14% during the construction of the model and with an error of 4.04% during the validation. The results illustrated on the aquagram reflect the tendency that authentic samples and samples containing minimal amounts of corn flour contain more hydrogen bonded water, as shown by the first harmonic range (~1,450 nm) of the O-H valence vibration, than samples counterfeited to a larger extent.

The NIRS-based method used in the research provides objective results quickly and with sufficient sensitivity. The results of the analyses help to gain more information about the characteristics of quality products, and to detect their genuineness and counterfeiting.

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