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Original Article

New technical possibilities for the determination of delayed fluorescence to study biological matter: Characterization of the measurement technique (FAS and EmSpec) and its application to eggs, wheat and kefir

Jenifer Wohlers[1,*], Matthias Rang[2], Philipp Bokatius[1], Gudrun Mende[1] and Peter Stolz[1]
[1]Forschungsinstitut, KWALIS gGmbH, Fuldaer Str. 21, D-36160 Dipperz, Germany.
[2]Naturwissenschaftliche Sektion, Goetheanum, Hügelweg 59, CH-4143 Dornach, Switzerland.

ABSTRACT

In this article, we describe some possible areas of application of a newly developed device (FAS-2) that measures light-induced delayed fluorescence and present its performance parameters. To demonstrate its application to food, measurements were carried out on wheat grains and on egg yolk from different organic and non-organic origins, as well as on kefir which was produced using different cultures and types of milk. The performance of the newly developed device FAS-2 was comparable to that of its predecessor, FAS-1, and it showed improved precision in differentiating the origin of egg yolk. The advantages of the newly introduced measuring mode of the FAS-2, which measures the emission with spectral resolution (EmSpec-mode), were explored by using kefir. It was shown that the freshness of kefir could be detected independently of other characteristics of the milk, such as the type of milk. We would like to point out that this method is also applicable in areas such as biology or the food sector and can be used, for example, in the assessment of food quality and authenticity.

KEYWORDS: delayed fluorescence, food quality, food origin/authenticity.

*Corresponding author: forschung@kwalis.de

1. Introduction

Fluorescence spectroscopy is often used to investigate the composition of organic substances or the origin of foodstuffs, whereby fluorescence in the short-term time range of 1-100 ns (prompt fluorescence, PF) is used. Long-term fluorescence (delayed fluorescence (DF)) in the time range of 0.01-60 s is less frequently used. However, previous studies have shown that it has the potential to detect differences in samples due to origin or processing. Studies on animal products such as eggs or milk [1-4], and on plant products, especially wheat grains [4-7], have shown that products from organic farming systems have a product-specific characteristic DF that differs from that of conventional products. Processing also changes the DF in a distinctive way, indicating changes in the structure of the organic matter, for example when cereals are processed into pops or flakes [8]. The origin and authenticity of foodstuffs are of interest to retailers (to detect fraud or misdeclaration), but also to consumers. Animal welfare is also becoming increasingly important [9].

Several approaches have been tested to verify the authenticity of organic eggs, including the analysis of fatty acid profiles, stable isotope ratios or carotenoid profiles [10, 11] as well as chromatographic fingerprints of high-performance

liquid chromatography with ultraviolet detection (HPLC-UV) [12a] or proton nuclear magnetic resonance (1H NMR) spectra [12b], but these parameters primarily reflect dietary effects. Whether these parameters can also capture husbandry aspects such as animal welfare has not been investigated independently of feeding effects. However, studies by Köhler [2] on DF of egg yolk have shown that FAS is a promising approach to address the issue of animal welfare in addition to dietary effects. In order to evaluate the performance of the newly developed measuring technique FAS-2 in comparison with the previously used FAS-1 device (as described in the Methods), measurements were carried out with both devices. Results of this initial comparison of the two devices for organic and non-organic eggs are shown below.

For plant products such as cereals, DF has also been used to study the differentiation of origin, particularly with samples from the DOK-trial [13] in which different cropping systems are compared. The impact of the cropping system on agronomic and quality parameters of the crops is well known [14-16]. Additionally, FAS measurements have been carried out on samples from this trial for several years, with consistent results linking quality to maturity characteristics [7]. It was of interest whether the newly developed FAS-2 would be able to achieve the same results as the well-established FAS-1, ensuring compatibility with previous measurements. This is particularly important because cereals make up an important segment of the organic market and are a frequently used crop in cropping system studies, and their handling is relatively simple compared to fresh organic matter, such as legumes.

Previously, some knowledge of the DF of milk from organic and non-organic origin was gained using the FAS-1 device [17, 18]. When using white light for excitation (with wavelengths ranging from approx. 200 to 800 nm), both farming-related factors like origin or diet become visible, as well as handling-related effects like ageing due to storage. Both types of effects are present simultaneously, are detected in combination, and cannot be separated from each other in white excitation mode. To investigate how the spectral properties of DF can be used to better separate the sample properties, we used the EmSpec mode of the FAS-2 device.

In this paper, we propose a new approach to detect DF excited by light absorption in the visible spectral range (VIS). The performance of the newly developed measurement device FAS-2 was evaluated by applying the FAS technique to egg yolk and wheat to assess its ability to differentiate between different origins, and to kefir to examine the device's newly introduced function to measure emission in spectral resolution (EmSpec).

2. Materials and Methods

2.1. Materials and handling of sample materials

Eggs from laying hens originated from a non-organic (conventional) farm and from an organic (biodynamic) farm. They were stored at 7 °C to keep them fresh until measurements were taken. Prior to the measurements, the temperature of the eggs was raised to 17 °C by placing them in the air-conditioned darkroom for one hour. Under weak red light, the yolk was manually separated from the whole egg. By inserting the tip of a syringe into the yolk, 8 g was taken off and transferred directly into a round quartz cuvette (d = 30 mm, h = 38 mm) where it was immediately measured by the FAS-1-device. This quartz cuvette was then transferred to the FAS-2 device where it was again immediately measured. Samples of organic and non-organic origin were measured alternately until six eggs per sample were examined.

Wheat from the DOK-trial was used (two field repetitions of each of the biodynamic and the conventional variant, harvested in 2009). At that time, in 2009, the grains had been equilibrated over silica gel at 30 °C for four weeks, measured with the FAS-1 device and subsequently stored in closed glass vessels until 2023. For the comparative measurements with the FAS-2, the grains were equilibrated again for 3 weeks at 30 °C over silica gel and returned to the glass vessels, which were transferred directly into the air-conditioned darkroom for the subsequent measurements. Under weak red light, the grains were placed in a round quartz cuvette (d30h50mm)

until it was completely full and then the measurement was taken by the FAS-1 device. Immediately afterwards, these grains were transferred into a petri dish (d75h20mm) and measured in the FAS-2 device. The arrangement in the measuring chambers is shown in Figure 1. Three batches (replicates) of each field repetition were measured.

Kefir was produced at a laboratory scale using two different kefir cultures: kefir tubers and freeze-dried kefir culture (cf. [19]). These were used to produce kefir from three different types of raw milk: cow's milk, goat's milk and sheep's milk. Measurements were performed on fresh kefir (day 1 after production) and on aged kefir (refrigerated at 8 °C for 10 days). Prior to the measurements, the kefir was placed in the dark room for 1 hour. For measuring, the kefir was gently shaken, and 40 g was poured into a petri dish made of quartz glass (d = 75 mm, h = 20 mm) and measured immediately afterwards with the FAS-2 device in EmSpec mode.

2.2. Methods

Overview: Fluorescence excitation spectroscopy was performed by using two different experimental setups, referred to as FAS-1 device and FAS-2 device. Each setup utilizes a halogen lamp for the excitation with visible light in eight different spectral regions. Photomultipliers are used to detect time-resolved delayed fluorescence. The difference between the setups is in their layout, including the direction of excitation and emission, along with several other specifications described below. For both setups, environmental data is continuously recorded to ensure that the observed results are not affected by external conditions. The measuring devices are positioned in an air-conditioned darkroom (15 ±1 °C, 40 ±5% R.H.) with weak red light, which was used to allow visibility while minimizing impact on the samples.

The well-established setup FAS-1 was conceptualized and developed by Strube in 1990 and has since been validated for vegetable samples by Strube and Stolz [5]. Its basic design follows the fluorometer with a right angle between the optical axis of light excitation and the optical axis of fluorescence detection. A halogen lamp (Xenophot 64640 HLX, 150 W 24 V G6.35 FCS, Osram, München, Germany) mounted in a computer-controlled projector (Novamat 130AF, Braun Photo Technik, Nürnberg, Germany) approximately

FAS-1
Wheat grains in quartz glass cuvette

FAS-2
Wheat grains in quartz glass petri dish

Figure 1. Measuring arrangement for wheat (for FAS-1 in cuvette, for FAS-2 in petri dish).

44 cm from the sample, is used to excite the sample. To realize excitation in spectroscopic resolution, the light beam can be filtered by seven different coloured glass filters (Schott, Mainz, Germany), namely RG695 (dr, dark-red), RG630 (re, red), OG590 (lr, light red), OG530 (ye, yellow), VG6 (gr, green), BG12 (bl, blue) and UG11 (UV, ultraviolet). Excitation without a filter is assumed to be white [wt]. Additionally, there are two separate 10% and 30% reflective neutral-density filters that can be used to reduce the excitation intensity. These filters can be used individually or combined when the sample's emission exceeds the photomultiplier tube's dynamic range, preventing its saturation. The sample emission is detected at a 90-degree angle to the excitation in the horizontal direction using a photomultiplier (EMI 9202, Thorn EMI Electron Tubes, Middlesex, England) with a 48 mm photocathode (Multialkali S20, Thorn EMI Electron Tubes, Middlesex, England). The photomultiplier is primarily cooled down to -30 °C by Peltier elements (MELCOR 1.4-71-045) and secondarily by water circulation. This setup is sensible to a wavelength region from 200 to 860 nm, with the photocathode achieving a maximum quantum efficiency of 31.2% at 275 nm. The single electron response (SER) is amplified (Discriminator amplifier EMI C604-A, Thorn EMI Electron Tubes, Middlesex, England) and counted by a data acquisition board (data translation DT2819). Measurements are conducted in single-photon-counting mode (internal noise: 13 counts per second at a PMT voltage of 1167V) and captured in consecutive intervals to obtain a time-resolved decay curve. For each measurement, the emitted photons are counted in either 100 milliseconds intervals for a total of 10 seconds, or 1.0 second intervals for 100 seconds. Pneumatically controlled shutters regulate the excitation duration and initiate the detection phase, which begins 0.2 seconds after the end of the excitation. The device was controlled by a PC (P 486 40 MHz, Intel), with software written in Modula-2, managing the measurements and data acquisition.

The newly developed setup FAS-2 was conceptualized and realized by Rang in 2023. A halogen lamp (OSRAM 64292 XIR) is used to induce excitation, modulated by a computer-controlled 18-slot filter wheel, which is fitted with 7 coloured glass filters and one ND filter identical in specifications to those used in the FAS-1 (see above). The halogen filament is focused on the shared end of a Y-shaped fiber optic light guide (2700 x UV 185/200/220 SiA – NA = 0.22/0.37 - L=1160 mm from CeramOptec). With its bifurcated ends attached to the top of the measuring chamber, the two light beams are directed towards the sample, with each beam entering at a 35-degree angle from the vertical axis. The optical axis of both ends meet at the position of the sample and illuminate it from different sides. The apertures of the two light beams and two photomultipliers are equipped with pneumatic shutters. In contrast to the FAS-1, the detection phase begins immediately after both excitation shutters are fully closed (twice as fast as FAS-1). The photomultipliers with a 48 mm photocathode (9202QMB with Voltage Divider C625AFP from ET Enterprises) are positioned in the measuring head at angles of 30 degrees to the vertical axis yet horizontally offset by 90 degrees (Figure 2). The distance from both photocathodes to the sample is 10.3 cm. This layout allows both photomultipliers to simultaneously measure the entire area of a planar sample (e.g., leaves). For spherical samples (e.g., apples), the shared measurement area is 67%, reducing the impact of local variations in the sample surface compared to the FAS-1, which has no shared sample area. Active cooling of both photomultipliers to -27 °C using 98% ethanol allows for low internal noise levels of 11 and 13 counts per second, respectively, at a PMT-Voltage of 1250V. The first photomultiplier operates within a wavelength range of 200-840 nm, achieving a peak quantum efficiency of 33.5% at 200 nm, while the second photomultiplier operates within the same range, achieving a peak quantum efficiency of 34.8% at 200 nm. The SERs are amplified (INA 02186 RF amplifier module) and acquired in single-photon-counting mode (counter card PMS-400A, Becker & Hickl, Germany) in consecutive intervals of 10 ms (with free choice of the length of interval between 2 to 65536) during a measuring period of 10 seconds (configurable between 250 ns and 100,000 s). Even with similar PMT types, the new

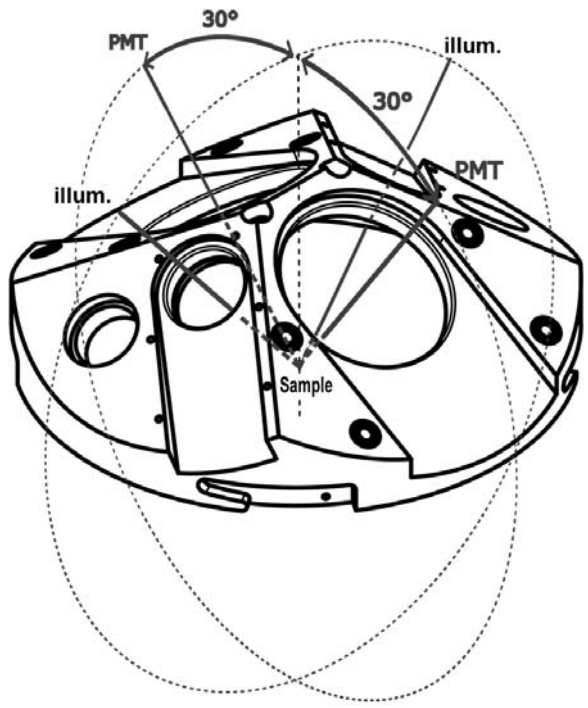


Figure 2. Construction view of the measuring head without attached modules. The illumination directions of the two glass fiber light guides (illum.) are indicated by the two straight lines that meet at the sample centre (Sample); the ellipse indicates the plane of illumination. The axis of optical detection by the photomultipliers (PMT) are indicated by the two straight lines between PMT and sample; the ellipse indicates the plane of detection. The angle between the vertical axis and the plane of detection is 30°, while illumination and detection are horizontally offset by 90° (not shown in this figure).

electronic amplification and acquisition signal path allows an approximately three times higher dynamic range compared to the FAS-1 setup.

EmSpec mode of FAS-2: The spectral distribution of sample's emission can be analysed by using different filters in front of the PMT. This is realized by an additional 9-slot filter wheel placed in front of each photomultiplier, which includes options for various filters (for the presented test measurements in use: LEE Nr. 299: 1.2 Neutral Density [ND], Nr. 027: Medium Red [dr], Nr. 019: Fire [re], Nr. 015: Deep Straw [or], Nr.139: Primary Green [gr], Nr. 119: Dark Blue [bl]) – as well an empty slot [wt] for neutral detection.

Additional features: The new setup features new software developed in Rust, with Tauri and React for the user interface, and an external MySQL database for efficient data management. These advancements enhance flexibility of measurement methods, as well as data processing, acquisition, and user interaction, contributing to the overall improved functionality of the setup. Furthermore, utilizing modern technologies ensures easier ongoing development and maintenance compared to the older technology stack used for the FAS-1 device.

2.3. Measurement settings and statistics

Egg yolk was excited with white light for 60 seconds, and emission was detected for 100.2 seconds after excitation in 1-second intervals (FAS-1 and FAS-2) and 0.01-second intervals (FAS-2). For comparisons, the data of the shorter measurement intervals was integrated into 1-second intervals, and the mean value of the time range from 60.2 s to 100.2 s was used for further statistics. A Student's t-Test was used to assess sample differences, with the coefficient of variation providing information about the data variation and the t-ratio indicating the dimension of the difference.

Wheat grains were excited for 5 seconds at each of the eight different wavelength ranges (seven-coloured filters and once without filter for white excitation, refereed to as FAS mode). Detection was done in 100 ms intervals. In cases of shorter measuring intervals in the FAS-2 (10 ms), the data was integrated into 100 ms intervals. In the FAS-2 device, a PMT-voltage of 1100V was used to adjust the PMT's sensitivity to the high emission intensity of the sample, and the blue excitation filter was paired with an ND-filter (LEE 299) to replicate the measuring conditions used in the FAS-1 setup for wheat, where the cereal setting (blue excitation filter combined with ND-filter from SCHOTT, NG 10) was applied. The emission after green excitation in relation to the emission after blue excitation (gr/bl) in the time range of 0.2 s to 0.3 s after excitation (parameter Mw1gr/bl) was used for further statistics. Tukey HSD test was used to assess sample differences, with the coefficient of variation providing information about the data variation and the t-ratio indicating the dimension of the difference.

Kefir was excited for 5 seconds and measured in FAS mode as well as in EmSpec mode (white excitation, emission filtered in 5 wavelength ranges). The emission in the time range of 1.0 s to 2.0 s after excitation was integrated and used for further statistics. A general linear mixed regression model was constructed using the software JMP 7.0 (SAS), with milk type (cow, goat, sheep), kefir type (tubers, culture) and freshness (day 1, day 10) as main factors; interactions were added for sensitivity analysis. Measured aliquots (two for day 1 and three for day 10) were assumed to be random. Results are presented for "white excitation – white emission" (FAS mode) and "white excitation – blue emission" (EmSpec mode).

3. Results and Discussion

3.1. Application to egg yolk: proof of origin

The fluorescence intensity of the samples differed according to the sample's origin, with higher emission in organic eggs (Table 1). This is in accordance with previous results [3]. The differences between the samples are more pronounced with the new measuring device FAS-2 (PMT-2), as indicated by a t-ratio of -10.9 (-10.5 for data obtained in 0.01s intervals) compared to t-ratio of -8.48 for the data from the established FAS-1 device (PMT-Z). For both devices, the data variation is higher for the organic sample and is therefore consistent with previous results [3].

To validate the comparability of the different measurement intervals possible in FAS-2, measurements taken with 10.0 ms intervals were compared with those with 1.0 s intervals. The count values were comparable and the fluctuation range of the data was the same. Only when the data of the shorter interval were integrated into 1.0 s intervals was the data variation of the conventional sample slightly increased. The measurement interval therefore had no relevant impact on the results. This indicates that the FAS-2 device provides very precise data acquisition, even with 100-times shorter measurement intervals (0.01 s) compared to the 1.0 s intervals used in FAS-1. Split values for organic and conventional samples have been published by Wohlers *et al.* [3]. The split value depends on the sensitivity of the measurement device. In the new device, the sensitivity can be changed by the PMT voltage and the input threshold (*it*) for the counter. The split value of 61.3 cts for R40w of the established device refers to a split value of 113.75 cts in the newly developed device under the used settings of 1250V and *it* = -0.0197 for the PMT-2. The fluorescence of the egg yolk declines after excitation in a characteristic way: the organic eggs emit at a higher level and decline less than the conventional eggs [3]. With the newly developed FAS-2 device, this slower decay of the organic eggs can be measured even more clearly. This is shown in Figure 3 and is the reason for the clearer differentiation in the parameter R40w in the time range of 60.2 s to 100.2 s.

These results indicate that the newly developed FAS-2 device is able to differentiate the eggs according to their origin in the same way as the well-established FAS-1 measurement device.

Table 1. Fluorescence parameter R40w (mean, SD, CV and t-ratio) of the organic and conventional samples for different measurement settings. PMT-Z is used in the FAS-1, PMT-2 in the FAS-2 device.

	Sample	**n**	**PMT * interval**		
			PMT-Z * 1.0s	**PMT-2 * 1.0s**	**PMT-2 * 0.01s**
Mean [R40w]	Organic	6	87.7 ± 9.58	176 ± 16.9	172 ± 15.2
± Std Dev	Conv.	6	53.6 ± 2.18	98 ± 4.4	99 ± 7.2
CV	Organic	6	10.90%	9.60%	8.84%
	Conv.	6	4.10%	4.50%	7.27%
t-ratio			-8.48	-10.9	-10.5

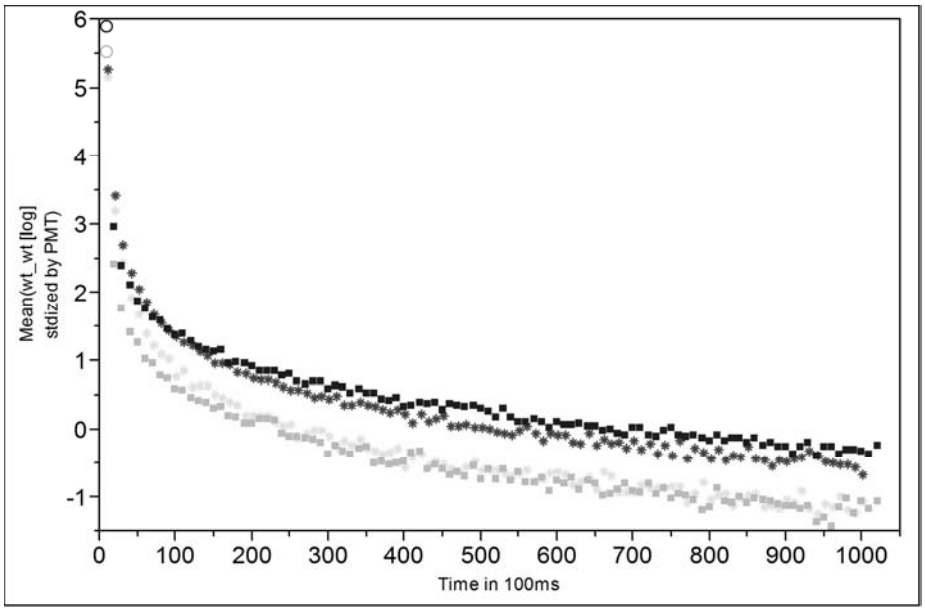


Figure 3. Mean decline curves of the 6 egg yolks of the organic (black symbols) and conventional (gray symbols) sample, for the FAS-2 (PMT-2, squares) and FAS-1 (PMT-Z, stars). Data log-transformed (to ensure normal distribution) and standardized by PMT (to adjust for different PMT sensitivity).

3.2. Application to wheat: differentiation of cultivation systems

The results of the measurements show that the differences between the cultivation systems are significant in both field replicates and for both devices (Table 2), although only three replicates were measured for each sample. The values are higher for the conventional samples, a characteristic of this type of sample [7]. The data from the well-established device FAS-1 shows the difference more clearly, with an F-ratio of 45.03 compared to 28.46 for the newly developed device FAS-2. Possible reasons for the less favorable degree of differentiation in the newly developed FAS-2 are manifold – the specific sensitivity of the PMTs and spectral quantum efficiency of the photocathodes have to be mentioned first, but the different optical densities of the ND-filters used are also likely to contribute. Additionally, the transmitted UV-radiation in the excitation light of the FAS-2 device is extremely low compared to the FAS-1, which also has an effect on the blue excitation, as the blue bandpass-filter (UG11) allows transmission of wavelengths in the UV range (270 to 380 nm). This fact also partly explains the different values of the parameter Mw1gr/bl (approximately 0.9 for FAS-1 and 3.5 for FAS-2). It has already been shown that wheat exhibits a relatively high fluorescence after excitation with UV radiation [5]. The lack of this spectral sub-component with the current heat absorption filter in the FAS-2 setup (Qioptiq Calflex 3000 SP) could be a major reason. Further technical developments for increased UV transmittance are in progress to improve the performance of the FAS-2 and achieve the same excitation specifications for blue as the FAS-1.

These results show that with the newly developed measurement deviceFAS-2 it is possible to differentiate the samples in a comparable way to the well-established FAS-1.

3.3. Application to Kefir, using EmSpec

The application of FAS in milk showed that the fluorescence intensity is affected by storage-related ageing. Storage time correlates with higher emission intensities, especially for fluorescence in the time range of 1 s or 2 s after blue and white excitation. However, this value is also influenced by the sample-specific emission intensity, which depends on various milk characteristics (e.g. the cow's diet [17]). To get a first idea of whether the

Table 2. Fluorescence parameter Mw1gr/bl (mean, SD, CV and t-ratio) of the biodynamic and conventional wheat sample for different measurement settings. PMT-Z is used in the FAS-1, PMT-2 in the FAS-2. Different letters indicate significant difference between the samples.

	Sample		**PMT * interval**	
Parameter Mw1gr/bl		n	PMT-Z * 0.1s	PMT-2 * 0.01s (1100V)
Mean ± Std Dev	Biodynamic_1	3	0.864 ± 0.0099 b	3.177 ± 0.0299 b
	Biodynamic_2	3	0.863 ± 0.0116 b	3.230 ± 0.0085 b
	Conventional_1	3	0.931 ± 0.0018 a	3.567 ± 0.0341 a
	Conventional_2	3	0.925 ± 0.0115 a	3.469 ± 0.1123 a
CV	Biodynamic_1	3	1.1%	0.9%
	Biodynamic_2	3	1.3%	0.3%
	Conventional_1	3	0.2%	1.0%
	Conventional_2	3	1.2%	3.2%
F-ratio			45.03	28.46

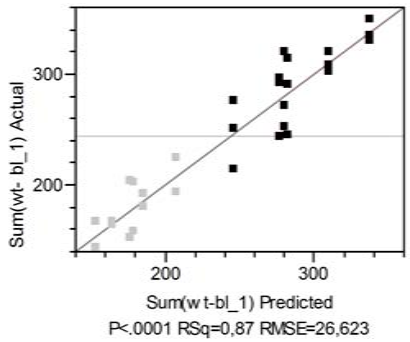
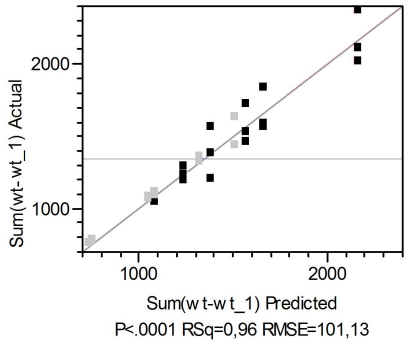
EmSpec mode of the FAS-2 setup could help to improve the discrimination of the sample properties, kefir samples were used as an example of a processed product, which was made of different milk types and measured fresh and aged. The results (regression analysis data are given in Table 3) show that in the usual measuring mode of the FAS (using the full spectral range of the PMTs without filter for detection, with white excitation and white detection), all factors of the regression model were represented in the measurement values, with milk type being the most important factor (indicated by the highest F-ratio), followed by freshness (Table 3). Differentiation due to freshness was uncertain in this case (correlation with R2 = 0.27). However, when using the EmSpec mode with the blue or green LEE filter in front of the PMTs, the time of ageing could be highlighted with an outstanding F-ratio in relation to the other characteristics of the samples (milk type, kefir culture). Differentiation due to freshness was possible in this case with R2 = 0.72. These results show that the additional use of a blue filter in front of the PMTs has improved the possibilities of showing ageing-related effects (freshness) as a single factor, separate from the other sample properties that become visible when measuring the entire range of wavelengths in the FAS-mode “white-white”. The EmSpec mode appears to be a promising tool for highlighting single sample properties.

4. Conclusions

The possibility of differentiating organic hens' eggs from non-organic ones improved when using the newly developed FAS-2 measurement device compared to the well-established FAS-1 device. In wheat grains from different cultivation systems, the response differences of the grains in their ability to react to excitation was detectable with both setups, whereby the FAS-2 setup showed a less effective differentiation of the variants, which is supposed to be related to its lower UV-content in the blue excitation light. Thus, even with slightly different geometry and illumination, it was possible to discern the origin of the eggs or the cultivation system of the wheat grains.

The performance of the FAS-2 is thus comparable to that of the FAS-1, but offers greater precision in differentiating between the origins of egg yolk

Table 3. General linear mixed regression model for white excitation and blue filter in front of the PMT (white-blue, EmSpec mode) and for white excitation without any filter in front of the PMT (white-white, FAS-mode). The model considered the main factors and their interactions as being culture, milk type and freshness. Measured aliquots (two for day 1 and three for day 10) were assumed to be random. Data represents the sum of emission in the time range from 1 s to 2 s after excitation. Fresh samples are shown in gray, aged samples in black. (* = p≤ 0.05; *** = p≤ 0.001).

		EmSpec mode (excitation-detection)		**FAS mode** (excitation-detection)	
		White-blue		**White-white**	
RSquare		0.87		0.96	
RSquare Adj		0.79		0.94	
Root Mean Square Error		26.6		101.1	
Mean of Response		244.4		1347.2	
Observations (or Sum Wgts)		30		30	
					
	Nparm	**F-ratio**	**Prob > F**	**F-ratio**	**Prob > F**
milk type [cow. goat. sheep]	2	5.87	*	119.87	***
kefir [tubers. freeze-dried culture]	1	1.29		22.83	***
freshness [day 1. day 10]	1	135.34	***	108.30	***
milk origin*kefir	2	3.38		4.81	*
milk origin*freshness	2	0.21		0.58	
kefir *freshness	1	0.44		6.93	*
milk origin*kefir *freshness	2	0.72		0.54	

and improved possibilities with the EmSpec application to emphasize sample characteristics such as the freshness of kefir over other characteristics such as the type of milk. It seems to be a promising method to measure delayed fluorescence of organic substances and thus to assess authenticity of origin or processing and handling. We note that this method is widely applicable to fields such as biology or food quality.

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CONFLICT OF INTEREST STATEMENT

No conflict of interest to declare.

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