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Multivariate Autofluorescence of Intact Food Systems

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1. Introduction

Public interest in food quality and production has increased in recent decades. This increase is related to changes in eating habits, consumer behavior, and the industrialization of the food supply chains. The demand for high quality and safety in food production calls for high standards in quality and process control, which in turn requires sensitive and rapid analytical technologies: sampling methods, sensors, and data analysis. In this context, fluorescence spectroscopy constitutes an interesting sensor technology, since several functionally important fluorescent substances are inherent to food systems including proteins, vitamins, secondary metabolites, pigments, toxins, and flavoring compounds.

Fluorescence spectroscopy is widely used in biological sciences due to its high sensitivity and specificity. The main research and applications in the field are usually carried out using specific fluorescence probes developed for selected problems, and the data analysis is often linear regression using a single wavelength. Typically, the feasibility of such analyses requires as a prerequisite extraction, chromatography, chemical labeling, or other sample separation steps prior to the fluorescence measurement. An alternative approach, which is the one covered by this review, is to measure the intrinsic fluorescence (autofluorescence) of the intact biological sample. Autofluorescence of intact biological samples is normally influenced by numerous analytes, and hence, numerous overlapping spectra make up the recorded signals. However, such an approach may increase the speed of analysis considerably and facilitates nondestructive analyses. The nondestructive mode of analysis is of fundamental scientific importance, because it enhances the exploratory dimension to the measurements, allowing for more complex relationships such as the effects of the sample matrix to be assessed.[1]

Intact foods are usually complex physical and chemical systems which in most cases include several intrinsic fluorophores and other phenomena which influence the recorded fluorescence signals. To handle the complex fluorescence signals obtained when analyzing intact food systems, chemometrics in the form of multivariate and multiway data analysis can be applied. The multivariate approach has proven beneficial in analysis of nonselective signals and specifically with respect to handling of interferents and as a diagnostic tool for detection of deviating samples (outliers).[2] Scientifically significant is the possibility of uniquely decomposing the complex signals from a series of 2D excita-

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Jakob Christensen (born 1971) graduated with an M.Sc. in food science in 1999 from the Royal Veterinary & Agricultural University in Copenhagen. He subsequently worked as a project coordinator of two European Union projects and was at the same time enrolled as a Ph.D. student at the university. His research was focused on autofluorescence of intact food systems with special emphasis on exploratory multiway studies. He was awarded his Ph.D. in 2005 at the Royal Veterinary & Agricultural University. Parallel to his Ph.D. studies he conducted research on exploratory data analysis applied to such diverse applications as wastewater, process analysis, biosensors, immunoassays, and fast instrumental spectroscopic methods. He is the author of 12 peer-reviewed scientific papers and holds a patent.

Lars Nørgaard (born 1964) graduated with an M.Sc. in chemical engineering in 1990 from the Technical University of Denmark, and he obtained his Ph.D. on the theme Flow Injection Analysis and Chemometrics in 1993 under the supervision of Dr. Carsten Ridder and Professor Elo H. Hansen. In 1995 he was tenured as an associate professor of exploratory data analysis and chemometrics at the Royal Veterinary & Agricultural University in Copenhagen in the group of Professor Lars Munck. His research is focused on chemometrics with applications in the areas of spectroscopy, analytical chemistry, food technology, process analysis, and medicine. Current research interests are model validation, graphic presentation of results from multivariate models, and classification methods for collinear multivariate data. He is the author of 45 peer-reviewed scientific papers and more than 20 proceedings, book contributions, reviews, and patents.

tion−emission fluorescence landscapes into the pure constituent signals using multiway chemometrics. We have coined this approach *mathematical chromatography* due to the fact that the separation (chromatography) of the constituent signals is performed mathematically (in computro).[3]

In this review we aim to provide a comprehensive overview of scientific activity concerning multivariate autofluorescence of intact food systems. The review is written with a special view of the current paradigm shift in industrial quality control by the introduction of process analytical technology where fluorescence sensors may provide valuable

Rasmus Bro (born 1965) studied mathematics and analytical chemistry at the Technical University of Denmark and received his M.Sc. in 1994. In 1998 he obtained his Ph.D. (Cum Laude) in multiway analysis from the University of Amsterdam, The Netherlands. Since 1994 he has been employed at the Royal Veterinary & Agricultural University in Copenhagen, and in 2002 he was appointed full professor of chemometrics. He has had several stays abroad at research institutions in The Netherlands, Norway, France, and United States. Current research interests include chemometrics, multivariate calibration, multiway analysis, exploratory analysis, experimental design, numerical analysis, blind source separation, curve resolution, Matlab programming, and constrained regression. In 2000 he received the 3rd Elsevier Chemometrics Award for noteworthy accomplishments in the field of chemometrics by younger scientists, and in 2004 he received the Eastern Analytical Symposium Award for Achievements in Chemometrics. He has authored 70 peer-reviewed scientific papers, 2 books on chemometrics, and more than 20 proceedings, book contributions, reviews, and patents.

Søren Balling Engelsen (born 1961) received his M.Sc. in FT-IR spectroscopy in 1988, and in 1992 he obtained a Ph.D. in molecular modeling from the Technical University of Denmark. In 1990 he was guest researcher at the Institute of Food Science, Cornell University, United States, and from 1993 to 1995 he was a postdoc at the Institut National de la Recherche Agronomique, Nantes, France. In 1997 he became associate professor in spectroscopy and molecular modeling at the Royal Veterinary & Agricultural University in Copenhagen. In 2004 he was appointed a full professorship in plant food science and is now the head of the Quality & Technology section. His primary research interests concern the development and application of nondestructive spectroscopic sensors for food analysis and quality control. His interests also include the technology chain *spectroscopy–molecular modeling–multivariate data analysis* with focus on solving problems related to biotechnology and functionality of plant-based hydrocolloids. He is the author of 85 peer-reviewed scientific papers.

complementary (to near-infrared) fingerprinting information of process streams and reactions. The focus will be on the multivariate mathematical approaches that are required to extract information from the complex signatures that are often the result of measuring autofluorescence of intact food

systems. After a brief introduction to the basic principles of fluorescence spectroscopy with focus on the phenomena affecting the intrinsic fluorescence of intact biological samples, the most relevant chemometric tools used in the evaluation of fluorescence data will be described. Subsequently, examples of current applications of autofluorescence measurements in different fields of food analysis will be given.

2. Fluorescence Spectroscopy

2.1. Basic Principles

Fluorescence (the name originates from the fluorescent mineral fluorspar) refers to cold light emission (luminescence) by electron transfer in the singlet state when molecules are excited by photons. The electromagnetic phenomenon *fluorescence* is a three-stage process that occurs in certain molecules called *fluorophores* or fluorescent dyes.[4] First, the fluorophore is excited to an electronic singlet state by absorption of an external photon ($h\nu_{ex}$). Second, the excited state undergoes conformational changes and interacts with the molecular environment in a number of different ways, including vibrational relaxation, quenching, and energy transfer. Third, a photon ($h\nu_{ex}$) is emitted at a longer wavelength, while the fluorophore returns to its ground state. The difference in energy or wavelength between the absorbed and the emitted photon is called the Stokes shift. The fluorescence *excitation* and *emission* of light typically appears within nanoseconds and is independent of temperature. A similar, but slower luminescence phenomenon is phosphorescence in which the photon goes through an intermediate excited triplet state where the afterglow lasts longer than microseconds and which is temperature-dependent. The molecular structure and environment is decisive for whether a compound is fluorescent. Fluorescence is often exhibited by organic compounds with rigid molecular skeletons, usually polyaromatic hydrocarbons and heterocycles. The less vibrational and motional freedom in the molecule, the greater the possibility that the difference in energy between the excited singlet state and the ground electronic state is sufficiently large that deactivation by fluorescence will occur.

Fluorescence is unique among spectroscopic techniques, because it is inherently multidimensional. A fluorophore needs a specific level of energy to be excited, and the subsequent emission energy corresponds to the difference between the excited and ground electronic singlet states. Each electronic state has several associated vibrational levels, which implies that excitation does not occur at only one single wavelength, but rather over a distribution of wavelengths corresponding to several vibrational transitions. While the deactivation of the excited state only occurs from the vibrational ground level, emission also occurs at several wavelengths as it may reach different vibrational levels in the electronic ground state. The result is that all fluorophores have independent and specific spectral excitation and emission profiles characterizing their unique fluorescent properties. These profiles can be measured as excitation and emission spectra or as a complete excitation−emission matrix (EEM), also known as fluorescence landscapes. The fluorescence spectra and the landscape of the aromatic amino acid tryptophan are depicted in Figure 1.

Besides the high specificity of fluorescence spectroscopy, the Stokes shift is fundamental to the sensitivity of the fluorescence measurements. The Stokes shift, which, in contrast to absorption spectroscopy, allows for emission photons to be detected against a low background, combined with efficient detectors in the visual range makes fluorescence spectroscopy a very sensitive analytical method with possibilities to measure down to parts per billion levels.

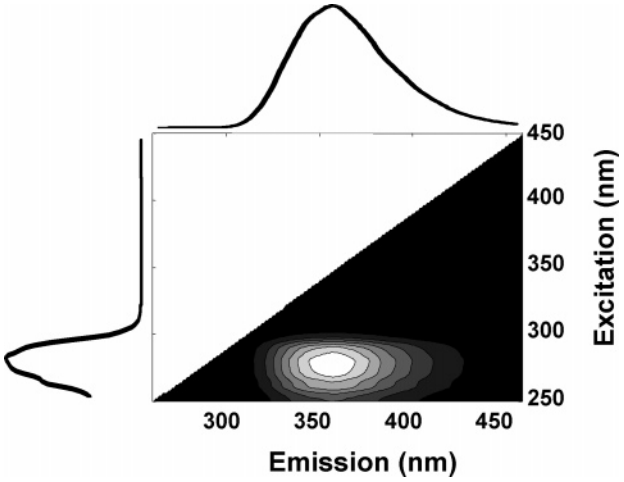

Figure 1. Fluorescence landscape (EEM) of 10^{-5} M tryptophan in water. The excitation (vertical) and emission (horizontal) spectral profiles are indicated with the surrounding curves.

2.2. Fluorophores in Food

Food contains a wide range of naturally occurring fluorescent compounds which are important for the nutritive, compositional, and technological quality. A comprehensive review of naturally occurring fluorescent compounds was made by Wolfbeis in 1985.[5] The review included several food-relevant fluorescent compounds such as aromatic amino acids, vitamins and cofactors, nucleic acids, porphyrins, flavonoids, coumarins, alkaloids, and myco- and aflatoxins. The archetypal application of fluorescence in analysis of food is the detection of aflatoxins in figs.[6] The aflatoxins are strongly fluorescent and will by excitation at 360 nm exhibit strong bright green-yellow fluorescence emission. This is the reason some greengrocers have blue light in their display shelves.

Recently, a Web-based food fluorescence library was made available at www.models.kvl.dk (September 2005) with the fluorescence characteristics of a variety of intact food samples as well as a list of food-relevant single fluorophores. In Table 1 and Figure 2 the fluorescence spectral properties of these selected fluorophores are presented.

Table 1. List of 11 Food-Relevant Fluorophores and Their Fluorescent Properties[a]

fluorophore	excitation λ_{max} (nm)	emission λ_{max} (nm)
phenylalanine	258	284
tyrosine	276	302
tryptophan	280	357
vitamin A (retinol)	346	480
vitamin B_2 (riboflavin)	270 (382, 448)	518
vitamin B_6 (pyridoxin)	328	393
vitamin E (α-tocopherol)	298	326
NADH	344	465
ATP	292	388
chlorophyll *a*	428	663
hematoporphyrin	396	614

[a] Data from the FoodFluor Database at www.models.kvl.dk.

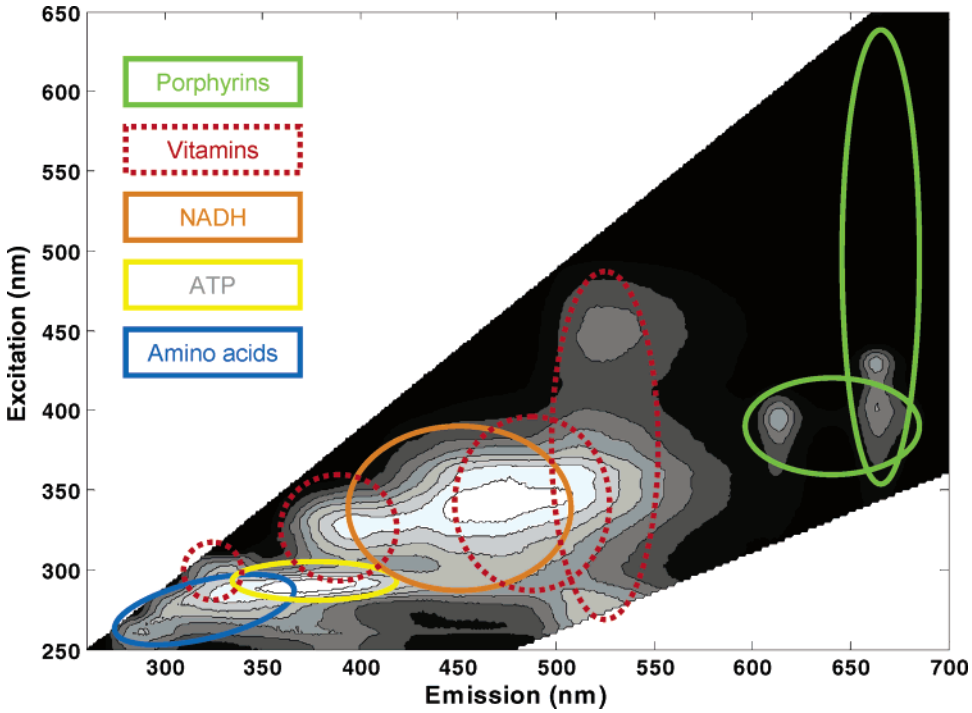

Figure 2. Fluorescence landscape map indicating the spectral properties of the selected 11 food-relevant fluorophores listed in Table 1. The presented contour plot makes up the sum of the normalized fluorescence landscapes of each of the 11 fluorophores.

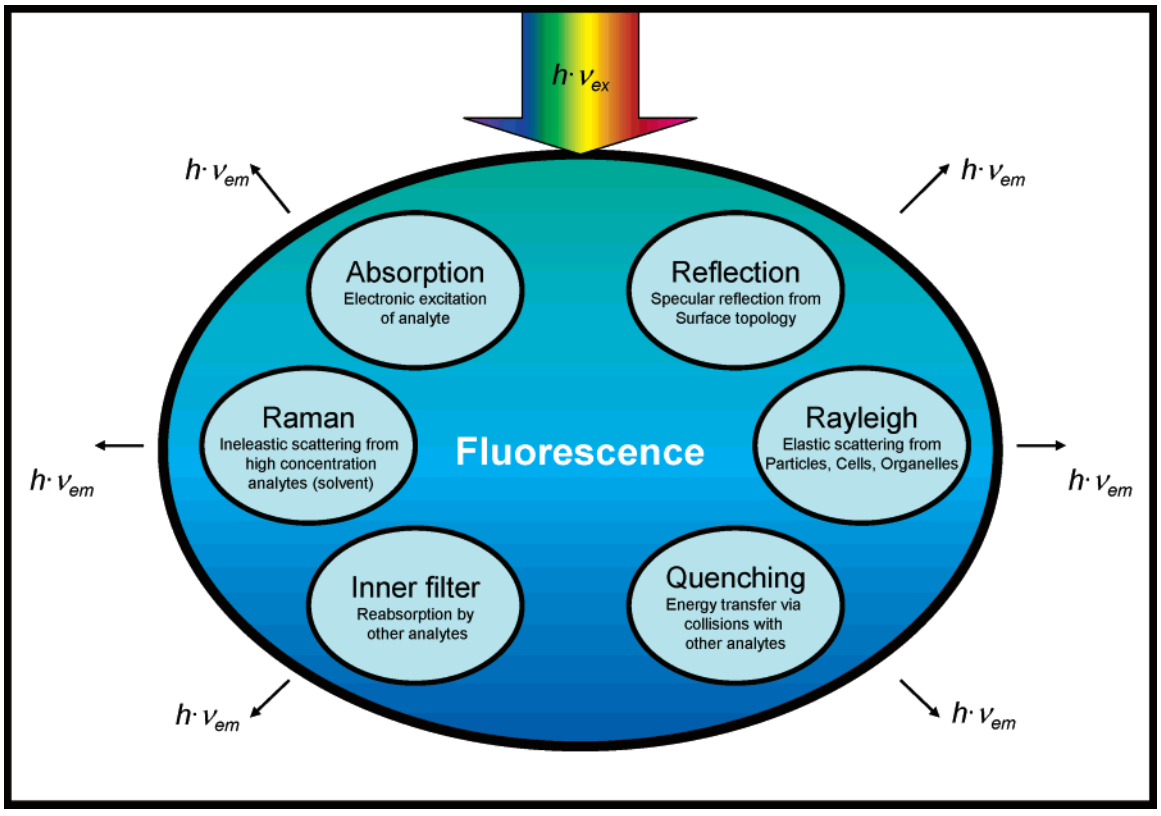

Figure 3. Diagram with an overview of factors affecting the fluorescence signal from complex samples.

2.3. Factors Affecting Fluorescence

An overview of factors that can affect the fluorescence emission signals is given in Figure 3. Several phenomena related to the nature of the food sample as well as the concentration and the local molecular environment of the inherent fluorophores will influence the fluorescence signal obtainable from biological samples. The effect of quenching, the concentration, and the molecular environment of fluorophores plus scatter phenomena will be briefly discussed in the following.

2.3.1. Quenching

Fluorescence quenching refers to any process which decreases the fluorescence intensity of a sample—a deactivation of the excited molecule by either intra- or intermolecular

interactions.[4] Quenching is either static or dynamic. Static quenching occurs when formation of the excited state is inhibited due to a ground-state complex formation in which the fluorophore forms nonfluorescent complexes with a quencher molecule. Dynamic or collisional quenching refers to the process when a quencher interferes with (deactivates) the behavior of the excited state after its formation. The excited molecule will be deactivated by contact with other molecules or by intra- or intermolecular interactions. Higher temperatures increase collisional quenching due to the increased velocities of the molecules. Resonance energy transfer can also be considered as a kind of dynamic quenching. Resonance energy transfer occurs when the emission spectrum of a fluorophore overlaps with the absorption spectrum of an acceptor molecule. The energy transfer does not involve emission of light, but rather a direct interaction between the donor and acceptor molecule, leading to a full or partial deactivation of the excited fluorophore (donor).

2.3.2. Concentration

The intensity of fluorescence depends on the concentration, the molar absorptivity, and the quantum yield of the fluorophore. Under ideal conditions in solutions with optical density below 0.05 AU,[4] the fluorescence intensity is approximately linearly proportional to the concentration of the fluorophore, according to

$$I_f = 2.3\varphi_f I_o \epsilon c l \quad (1)$$

where I_f is the fluorescence intensity, φ_f the quantum yield, I_o the intensity of the incident light, ϵ the molar absorptivity, c the molar concentration of the fluorophore, and l the optical depth (or path length of the cell) of the sample. The fluorescence signals are ideally additive in mixtures; i.e., the overall fluorescence signal of a given sample can be expressed as the sum of the fluorescence contribution from each of the inherent fluorophores. However, in complex mixtures such as intact food samples, the fluorescence may not be additive due to quenching phenomena and interactions with the molecular environment of the fluorophore.

2.3.3. Molecular Environment

The polarity of the local environment of a fluorophore influences the emission of especially polar fluorophores. In more polar environments, fluorophores in the excited state will relax to a lower vibrational energy state before emission, resulting in emission at lower energies, i.e., longer wavelengths. Shifts in emission spectra of fluorophores can be observed when comparing identical fluorophores in different solvents or as residues in different macromolecules. Figure 4 illustrates such an example, comparing the emission spectra of tryptophan from three different milk protein fractions. The observed emission is shifted according to the position and exposure (to the surroundings) of the tryptophan residues in the proteins. Clearly, the emission peak of the least structured protein, in this case casein, is shifted more toward longer wavelengths, indicating the most exposed in the surrounding polar environment (water).

Also pH and temperature strongly affect the fluorescence signal. With dissociation or protonation caused by pH changes, the rates of nonradiative processes competing with fluorescence can be altered and thereby affect the quantum yield of fluorescence emission. Temperature primarily affects fluorescence through its impact on dynamic quenching.

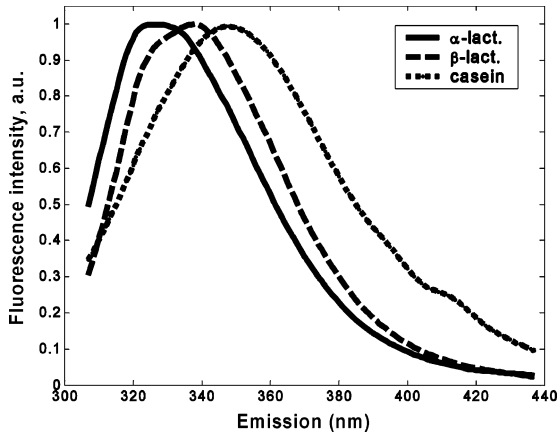

Figure 4. Normalized fluorescence emission spectra (excitation wavelength 295 nm) of a solution of milk proteins in water: α-lactalbumin, β-lactobglobulin, and casein. Concentrations correspond to approximate tryptophan concentrations of 10^{-5} M. Casein is from bovine milk and a mixture of α-, β-, γ-, and κ-casein.

2.3.4. Scatter

Scattering and reflection of the incident light has a substantial effect on fluorescence measurements, with respect to both the optical depth of the sampling and the obtained fluorescence signal. The most important parts of the scattered light can be divided into elastic Rayleigh scatter and inelastic Raman scatter. For Rayleigh scatter, the wavelengths of the scattered light are the same as those of the incident light, and in principle this type of scatter should not interfere with fluorescence emission, as these spectral areas can be disregarded when analyzing the fluorescence landscape. This is illustrated in Figure 1, where only emission wavelengths higher than 10 nm above the excitation wavelength are included. However, when working with instrumental setups with large bandwidths, Rayleigh scatter can constitute a significant interference to fluorescence emission from fluorophores with small Stokes shifts. Raman scatter is related to vibrational states of the bulk substances in the sample and reflects a constant energy loss. Raman scatter can in many cases be neglected because of its weak contribution to the fluorescence signal. Alternatively, corrections of the fluorescence signal can be performed either by subtracting the pure solvent/background scatter contribution or by specifically addressing the scatter in the modeling of the fluorescence data.[7,8]

2.4 Practical Issues Related to Sampling of Intact Food

In an ideal sample system for fluorescence measurements, several conditions need to be fulfilled: (i) The concentration range of the fluorophores must be at the appropriate level to be approximately linearly related to the fluorescence intensity. (ii) The fluorescence signals from each of the inherent fluorophores must be independent of each other. (iii) The signal contributions from interferents such as absorbing species and quenching phenomena must be insignificant compared to the fluorescence signal. Such conditions *cannot* be fulfilled in most intact food samples where the concentration ranges of the inherent fluorophores are not adjustable and several absorbing and quenching species

concurrently exist. Instead the complex fluorescence pattern from intact food systems can be approached pragmatically and considered as a spectral fingerprint of the sample based on its fluorescence characteristics as well as its absorbing and quenching abilities.[1] Using this approach, classifications and indirect correlations to quality parameters can be assessed.

For opaque intact food systems fluorescence will normally be measured using front-face illumination and, for example, a 60° or near-180° (optical probes) fluorescence collection to avoid specular reflection. When measuring autofluorescence of opaque intact food systems, primarily the surface is measured and most of the incident light will be absorbed near the surface of the sample; i.e., a short optical depth is obtained. This is essential for obtaining a linear relationship between fluorescence intensity and analyte concentration, according to eq 1. However, when measuring in front-face mode, the amount of scattered and reflected light reaching the detector will increase due to a higher level of reflection from the surface of the sample. The most important thing to bear in mind when measuring autofluorescence of opaque intact food systems is that it is a surface measurement. Whether the surface is viscous (honey), layered (fruits), or fibrous (meat) does not really matter, as long as one remembers that the measurement only represents the surface. It is thus critical to investigate whether the sample surface is representative for the quality attribute that is desired measured.

The literature is scarce on the important sample−apparatus interface in fluorescence spectroscopy for opaque anisotropic samples. However, it is our experience that orientation of heterogeneous and anisotropic food systems with respect to the measurement geometry is normally negligible and that instrumental uncertainty and sampling uncertainty can be accounted for. There is no need a priori to consider anisotropic (heterogeneous) systems difficult to sample. Sampling uncertainty is a matter of representativity and reproducibility, and it can be tested and validated using multivariate principles (independent test set validation and replicate analysis).

As mentioned in the Introduction, this review is written with a view of the current paradigm shift in industrial quality control by the introduction of process analytical technology. In the full implementation of this approach, fluorescence sensors need to measure directly on the process line or in the batch process and sampling representativity will be only dependent on the classical sampling issues such as the required mass reduction of the dynamic process flow passing the sensor optics. It is noteworthy that industrial on-line fluorescence sensors are highly feasible, as the wavelengths of the visual illumination and the fluorescence signals with practically no loss can be transmitted over long distances using quartz fibers, making several measuring points possible using a single spectrometer. On-line measurements include optical sensors in contact with the process streams or placed inside batch reactors as well as diverted side streams passing through the measurement apparatus. In the latter case, provided that sampling representativity is adequate, destructive sample handling such as flow injection dilution is possible.

This would certainly be the case if the food system to be measured is a transparent or semitransparent liquid, in which case the most common way to record fluorescence is in 90° geometry using a flow cell or a standard cuvette. Since the concentration of fluorophores in transparent food systems might not be at an optimal level, dilution of the food sample

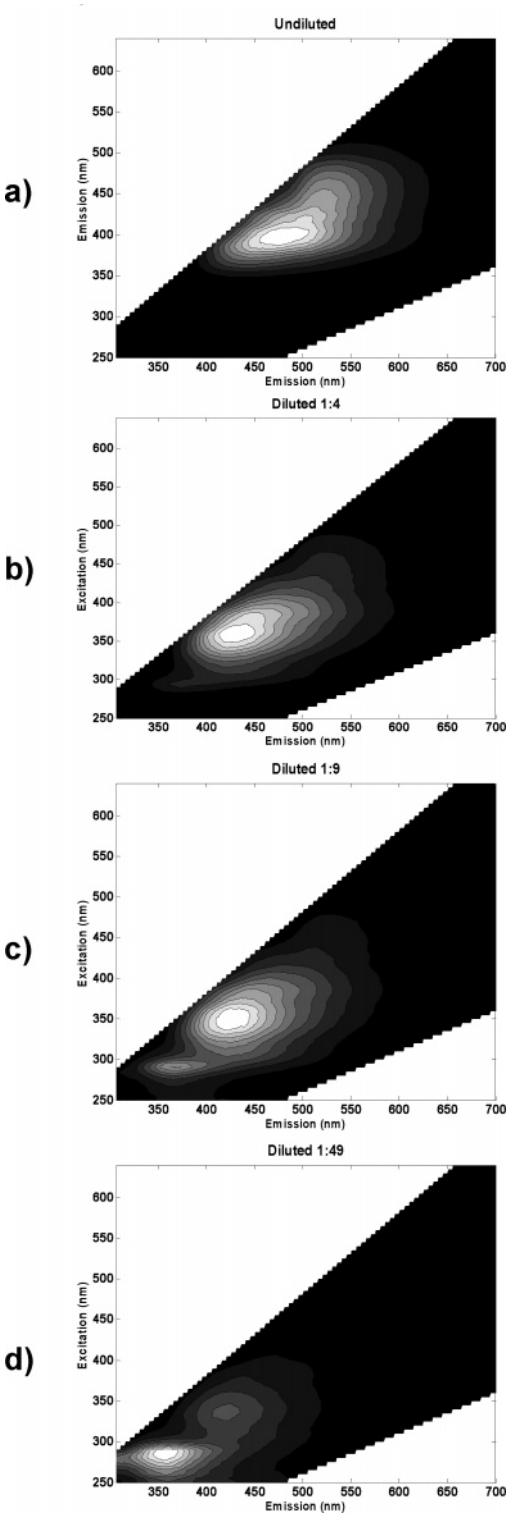

Figure 5. Fluorescence landscapes of a dilution series of beer (Carlsberg lager) samples: undiluted (a), diluted 5 (b), 10 (c), and 50 (d) times (d). Fluorescence measurements were recorded in quartz cuvettes with right-angle sampling geometry on a Perkin-Elmer LS50B instrument with settings according to the FoodFluor database at www.models.kvl.dk. Contour plots were normalized according to the maximum intensity (white) of the undiluted sample.

(if possible) is an option. However, it should be kept in mind that the dilution may change the concentration of other relevant fluorescent species below or close to the detection limit and that dilution may change interactions of the intact food matrix. In Figure 5, the fluorescence landscape of a beer sample at different dilution levels illustrates that

different fluorophores are expressed differently at different dilution levels in a nontrivial fashion. For the undiluted beer, fluorescence emission from excitation at 400−500 nm is dominating, which is probably due to polyphenolic compounds[9] and riboflavin.[10] Upon dilution with water, the fluorescence pattern of the beer completely changes until it is diluted 50 times, from which level the fluorescence pattern stabilizes and depends linearly on the dilution. When the sample is diluted 10 times, the distinct fluorescence from tryptophan dominates.[9] Such conditions are inherent to fluorescence analysis and thus inevitable when working with intact food samples.

3. Data Analysis

3.1. Fluorescence Data Structure

Fluorescence is inherently multidimensional, because the fluorescence emission process contains a wealth of independent information that is related to the fluorophore and its surroundings. Multidimensional fluorescence signals recorded from a sample can conveniently be presented as a matrix of fluorescence intensities as a function of excitation and emission wavelengths such as the fluorescence landscape presented in the contour plot in Figure 1. The fluorescence landscape or excitation−emission matrix of each fluorophore can ideally be described as a function of a concentration-dependent factor, α, and its excitation, $\mathbf{b}(\lambda_{ex})$, and emission, $\mathbf{c}(\lambda_{em})$, characteristics. Thus, the overall fluorescence EEM can be described according to eq 2, where i is used to

$$\mathrm{EEM} = \sum_{i=1}^{n} \alpha_i \times \mathbf{b}_i(\lambda_{ex}) \times \mathbf{c}_i(\lambda_{em}) \qquad (2)$$

enumerate the fluorophores and n is the total number of fluorescent species present in the sample. An important assumption is that the excitation and emission spectra are chemically independent of one another. Given several samples with different concentrations of the fluorophores, eq 2 can under ideal conditions be extended with an additional dimension to become trilinear.

3.2. Chemometrics

Complex problems need multiple variables to be adequately described. Spectroscopic data contain a large amount of highly correlated data from neighboring wavelengths. Multivariate data analytical tools such as principal component analysis (PCA)[11,12] and partial least squares (PLS) regression[13] have proven to be powerful methods for mathematical extraction of the dominant latent data structures of such collinear spectroscopic data. The use of multivariate techniques to explore and analyze the fluorescence signals from complex samples can be considered part of the field of chemometrics, founded by Bruce Kowalski[14] and Svante Wold[15] in the early 1970s.[16] Chemometrics has been defined as the "chemical discipline that uses mathematics, statistics and formal logic (a) to design or select optimal experimental procedures; (b) to provide maximum relevant chemical information by analyzing chemical data; and (c) to obtain knowledge about chemical systems".[16] Multivariate so-called latent variable methods are intrinsically more robust, for instance, toward peak shifts than univariate methods, because such multivariate methods use areas under whole curves (called scores in chemometrics) rather than just a single wavelength intensity. Such areas are much less influenced by moderate peak shifts than single wavelengths. Additionally, robustness is also obtained from the general noise reduction obtained from using the above-mentioned areas.

3.3. Multivariate Analysis of Fluorescence Data

Multivariate data analysis of fluorescence data can be approached in two different ways: (1) Analysis of fluorescence data arranged in data matrices. Typically, each row in the data matrix holds the data for one sample and each column corresponds to a specific excitation−emission wavelength combination. (2) Multiway analysis of data with variables in more than one dimension. Typically, every sample is a matrix (EEM), and gathering several matrices from several samples leads to a three-way box of data which is called a three-way array.

Traditional multivariate analysis of fluorescence data is usually performed on a series of either emission or excitation spectra contained in a matrix. Sometimes even whole sets of EEMs are rearranged into a matrix and analyzed with multivariate analysis. The most common data analytical approaches are PCA, used for exploring, visualizing, and mining the data, and PLS regression, used for building quantitative models, e.g., for predicting specific concentrations from the measured spectra. The application of multivariate data analysis in the evaluation of fluorescence spectroscopic data was first proposed in 1982 in a study describing the botanical constituents of wheat in wheat milling fractions.[17] PLS regression was successfully used to correlate emission spectra from intrinsic fluorescence of wheat milling fractions to the concentration of different botanical constituents in the flour, probably on the basis of the fluorescence signal from ferulic acid, tryptophan, and riboflavin.

3.4. Multiway Analysis of Fluorescence Data

Multiway data analysis refers to multivariate data analysis performed on data arrays which are three-way or even higher way as opposed to two-way data. In the case of fluorescence spectroscopy, three-way data analysis can be applied, for instance, when the fluorescence intensity as a function of samples, excitation wavelengths, and emission wavelengths is presented in a three-way data array. An example of a multiway model for decomposition of a trilinear data array is parallel factor analysis (PARAFAC).[18,19] The principle behind PARAFAC decomposition is to minimize the sum of squares of the residual, e_{ijk}, as indicated in eq 3, based on

$$x_{ijk} = \sum_{f=1}^{F} a_{if} b_{jf} c_{kf} + e_{ijk} \qquad (3)$$

a least-squares algorithm. The element x_{ijk} represents the data for sample i in variables j and k of the two different variable dimensions. The three-way data array is thus decomposed into a set of sample scores, a_{if}, loadings for the emission mode, b_{jf}, and loadings for the excitation mode, c_{kf}. The rank of the PARAFAC model is given by the number of factors, F, needed to describe the systematic variation in the data array. A graphical presentation of the decomposition is shown in Figure 6, where the three-way data array $\mathbf{X}$ is decomposed into scores and loadings, $\mathbf{a}$, $\mathbf{b}$, and $\mathbf{c}$, using two factors (or PARAFAC components), leaving the unmodeled part of $\mathbf{X}$

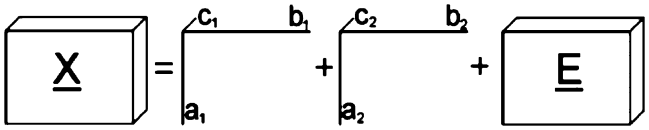

Figure 6. Principles of a PARAFAC decomposition of a three-way data array, **X**, into two factors of **a**, **b**, and **c** loading vectors.

in the residual array, **E**, which ideally only contains unsystematic noise.

The decomposition of three-way data gives a mathematically unique solution for a given number of components. Thus, there are no mathematical ambiguities in the solution except trivial scale and order issues. Therefore, if the PARAFAC model is also a description of the chemically meaningful structure, the parameters of the model will have a chemical interpretation. Specifically, each PARAFAC component will be an estimate of the contribution from one fluorophore, and this estimate is given by a score vector containing the relative concentrations, an emission loading being an estimate of the emission spectrum and an excitation loading being an estimate of the excitation spectrum. Thus, the PARAFAC model can be used for a unique decomposition of the fluorescence data from a complex sample set into a number of PARAFAC components corresponding to the number of fluorophores present in the samples. The PARAFAC analysis thereby facilitates the analysis of fluorescence measurements of complex biological samples, especially in exploratory situations when the fluorescence phenomena of the samples a priori are unknown. In the data analysis, the relative concentration of each of the present fluorophores in the mixture can be determined, and the excitation and emission loadings can be used for identification of the fluorophores.

Several other algorithms have been suggested for decomposition of trilinear data, as recently compared and reviewed by Tomasi and Bro in 2006.[20]

The advantages of recording and analyzing fluorescence landscapes in the investigation of a sample with multiple fluorescent components was introduced by Weber in 1961[21] and further explored and developed by Warner and coworkers at the University of Washington in the late 1970s.[22−25] In a series of papers, they demonstrated the principles of utilizing the experimental emission−excitation matrix in quantitative analysis of multicomponent samples for determining the number and spectra of the emitting components in the sample. Ho et al.[26−28] and others from the same research group[29−31] continued this work and developed a so-called rank annihilation factor analysis of fluorometric data to obtain a decomposition similar to eq 3. In these first studies, the multiway approach was only applied and explored on simulated fluorescence data and fluorescence measurements on model solutions. Later, multiway analysis was applied on biological samples to resolve the fluorescent components in spinach chloroplasts and green algae[32] and plant pigment complexes.[33]

An example of a PARAFAC decomposition of fluorescence data obtained from an intact food system is shown in Figure 7, based on a series of fluorescence landscapes of yogurt samples measured throughout a storage experiment.[34] The obtained fluorescence signal is decomposed into three factors, or PARAFAC components, that describe each of the fluorescence phenomena present in the yogurt samples. Scrutiny of the excitation and emission spectra of each of the resolved components can then be used for identification and understanding of the present fluorophores, which are suggested to be riboflavin, tryptophan, and an oxidation product. As the PARAFAC method is only well suited for "ideal" problems, it is fairly common to observe that one unidentified component could be a conglomerate or a distributed class of substances of more complex nature. In such cases, further identification can be pursued in more traditional manners.

When working with intact food samples, the conditions may not be ideal to obtain perfectly trilinear data with respect to concentration levels and possible quenching effects, as indicated by the fact that the obtained and resolved fluorescence signal from riboflavin (the first factor) in this case only seems to have two excitation peaks (at around 370 and 450 nm). In pure solutions, strong absorption from riboflavin will also appear at around 270 nm (cf. Table 1 and Figure 2), which is not evident in the yogurt study, probably due to competition with the strong absorption of this light by tryptophan.

4. Food Studies

As an introduction to autofluorescence from intact food systems, a few examples of food fluorescence are shown in Figure 8, where tentative chemical assignments can also be made. For the milk sample, the observed dominating fluorescence for excitation wavelengths below 300 nm can be assigned to protein fluorescence, and the fluorescence emission around 520 nm to riboflavin.[35,36] The fluorescence signal with excitation/emission maxima around 325/425 nm has previously been assigned to vitamin A.[37] The remaining peak around 360/440 nm corresponds in wavelengths to previous literature assignments for dairy products of oxidation products,[34] a Maillard product,[38] and NADH.[39] Tryptophan fluorescence also seems dominant for the wheat flour sample, from which also a broad peak emission from 400 to

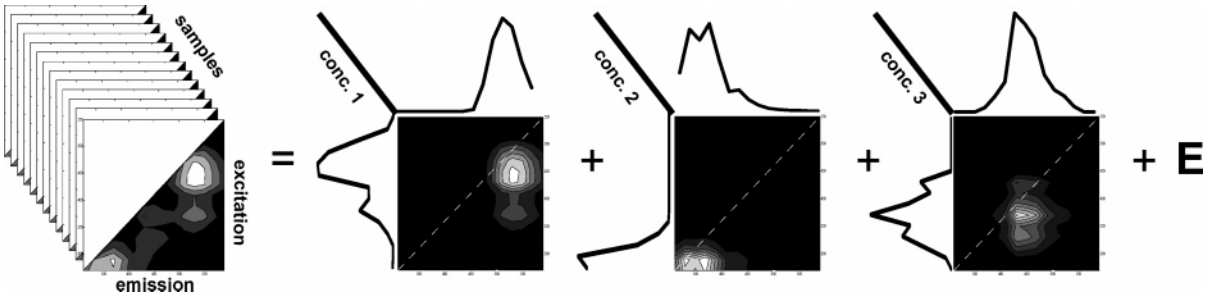

Figure 7. PARAFAC decomposition of fluorescence landscapes from yogurt samples into three factors. Curves in the vertical direction depict the loading vector for each of the factors; curves in the horizontal direction show the emission loading profiles. The three contour plots express the product of the excitation and emission loadings; fluorescence is only measured below the dotted line. Data were taken from ref 34.

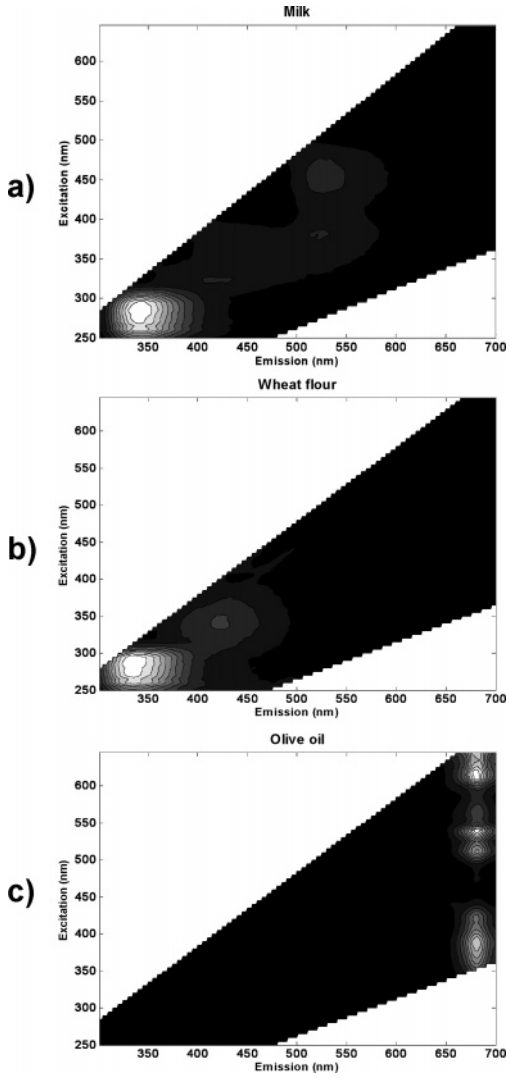

Figure 8. Fluorescence landscapes of autofluorescence recorded directly from intact food samples: (a) milk (1.5% fat), (b) wheat flour, and (c) extravirgin olive oil taken from the FoodFluor database at www.models.kvl.dk. Front-face fluorescence measurements were recorded in quartz cuvettes with 60° sampling geometry on a Perkin-Elmer LS50B instrument. Contour plots were normalized according to the maximum intensity (white).

500 nm, upon excitation around 350 nm, is observed. This is in agreement with a previous study[40] on the autofluorescence of cereal flours. The autofluorescence of neat olive oil expresses a completely different fluorescence pattern dominated by fluorescence emission in the visible spectral range. The most intense emission is observed just below 700 nm, corresponding to the fluorescent properties of chlorophyllic compounds according to Table 1.

Particularly in the past decade, the number of application studies of autofluorescence and chemometrics in analysis of intact food has increased. The following review of these application studies will be divided into subgroups according to the food products meat, fish, dairy products, edible oils, cereals, sugar, and fruit and vegetables. The categories were chosen to compare studies of similar products, and the division was also based on the number of published studies in each of the fields. Table 2 gives an overview of the studies in each application area, including literature references separated according to assigned fluorophores. Some of the assignments are questionable and will be discussed in the text.

4.1. Meat

Studies on applications of autofluorescence from meat have primarily been focused on measurements of collagen in connective and adipose tissues, but protein fluorescence and suggestions for some fluorescent oxidation compounds have also been reported.

Autofluorescence for analysis of meat was first proposed in 1986 in a patent[41] suggesting a method for quality control of meat and fish products based on their intrinsic fluorescence characteristics. The method was based on excitation at 340 nm and the fact that bone, cartilage, connective tissues, and meat possess different fluorescent properties. Only weak fluorescence signals are obtained for pure meat at this excitation wavelength, whereas the nondesired substances (fat, bone, cartilage, and connective tissue) all give a considerable fluorescence emission signal. The emission spectra for these compounds are different in shape, but they all have a peak with a maximum at 390 nm and a shoulder peak with emission at 455 nm (bone, cartilage, and connective tissue) or 475 nm (fat), which can probably be assigned to different types of collagen and NADH.

Since 1987 Swatland has written a series of papers on different aspects of the autofluorescence of meat.[42] His work was focused on measuring collagen and elastin fluorescence from the connective tissues in meat using excitation at 365 nm. The obtained autofluorescence signals of various meat samples were correlated to several sensory-related quality parameters such as gristle content in beef,[43] skin content and processing characteristics of poultry meat slurry[44] and turkey meat,[45,46] and palatability,[47] chewiness,[48] and toughness[49] of beef. All these correlations can be considered as indirect analyses due to the fact that these quality parameters are related to the amount and distribution of connective and adipose tissue in the meat. Swatland has also investigated technical aspects of the fluorescence measurements and instrumentation (backscatter, reflectance, direction of light), leading to the development of on-line meat probes based on fiber optics[50−53] with simultaneous measurements of reflectance.[54] Most of these studies were carried out with a univariate data analytical approach; univariate regression models were calculated between the desired quality parameters and single wavelengths or extracted fluorescence peak features. However, multiple linear regression of a few parameters was applied using a stepwise selection procedure,[48,49] pointing toward a more multivariate approach.

Egelandsdal, Wold, and co-workers later applied the bilinear methods PCA and PLS in evaluation of autofluorescence emission spectra of meat obtained from selected excitation wavelengths in the UV region between 300 and 400 nm. In their work, fluorescence emission spectra assigned to various types of collagen in meat products were found to correlate with tensile properties,[55] tenderness,[56] and water-holding capacity,[57] and recommended for quantification of connective tissue and collagen.[58,59] Fluorescence emission spectra assigned to fluorescent oxidation products have been found to correlate with lipid oxidation[60,61] and rancidity[62] of meat. Moreover, tryptophan fluorescence (excitation 290 nm) has been correlated to the texture of meat emulsions and sausages[63,64] and meat tenderness.[65] Also autofluorescence images reflecting the collagen fluorescence have been used for quantification of the intramuscular fat content and connective tissue in beef[66,67] as well as for mapping of the lipid oxidation in chicken meat.[68] As an example of a more peculiar meat application, the presence

Table 2. Overview of the Literature Survey on Autofluorescence Studies of Intact Food

fluorophore	meat	fish	dairy products	edible oils	cereals	beer	sugar	fruit and veg.
amino acids	Skjervold, 2003[67] Møller, 2003[70] Allais, 2003−4[63,64]	Dufour, 2003[73]	Dufour, 1997−2001[37,76] Birlouez-Aragon, 1998[38] Herbert, 1999−2000[77,86] Lopez, 2001[78] Mazerolles, 2001[79] Leclere, 2001[93] Christensen, 2003−5[34,95] Becker, 2003[36] Karoui, 2003−5[82,83,88−92] Kulmyrzaev, 2005[39,80] Garimelle Purna, 2005[81]		Jensen, 1982[17] Kissmeyer-Nielsen, 1985[103] Zandomeneghi, 1999[40] Jensen, 2004[110]	Apperson, 2002[113] Sikorska, 2004[10] Christensen, 2005[9]	Norgaard, 1995[125,126] Munck, 1998[1] Bro, 1999[129] Baunsgaard, 2000−1[127,131−133] Ruoff, 2005[128]	
collagen	Jensen, 1986[41] Swatland, 1987−2003[42−54,134,135] Egelandsdal, 1996−2005[55,56,59] Wold, 1999[58,66] Brøndum, 2000[57] Skjervold, 2003[67]	Jensen, 1986[41] Andersen, 2003[72]						
chlorophyll		Pedersen, 2002[74]	Wold, 2005[75]	Engelsen, 1997[96] Kyriakid is, 2000[97] Pedersen, 2002[74] Diaz, 2003[98] Guimet, 2004[122,136] Sikorska, 2004−5[100,101] Zandomeneghi, 2005[99]				Franck, 1969[114] Schreiber, 1975[115] Yamada, 1996[116] Song, 1997[117] Cohen, 1998[118] Moshou, 2003[121] Bron, 2004[119] Codrea, 2004[120]
ferulic acid					Jensen, 1982[17] Kissmeyer-Nielsen, 1985[103] Symons, 1991−6[105−108] Ram, 2004[109]			
Maillard products			Birlouez-Aragon, 1998[38] Léclere, 2001[93]				Baunsgaard, 2000−1[127,131−133]	
NADH	Jensen, 1986[41] Brøndum, 2000[57]	Jensen, 1986[41] Dufour, 2003[73]	Kulmyrzaev, 2005[80]			Sikorska, 2004[10]		
oxidation products	Wold, 2000−2[60,62,68] Møller, 2003[70] Olsen, 2005[61]		Christensen, 2003−5[34,9 5] Becker, 2003[36]	Engelsen, 1997[96] Sikorska, 2004−5[100,101] Guimet, 2004[122,136]	Jensen, 2004[110]			
polyphenols				Sikorska, 2004−5[100,101] Zandomeneghi, 2005[99]		Apperson, 2002[113] Christensen, 2005[9]	Baunsgaard, 2000−1[127,131−133] Ruoff, 2005[128]	
pyridoxine (vitamin B_6)						Sikorska, 2004[10]		
retinol (vitamin A)			Dufour, 1997−2001[37,85] Herbert, 2000[86] Christensen[95] Karoui, 2003−5[88−92] Kulmyrzaev, 2005[80]					
riboflavin (vitamin B_2)			Wold, 2002[35] Christensen, 2005[34] Becker, 2003[36] Mortensen, 2003[94]	Zandomeneghi, 2005[99]	Zandomeneghi, 2003[104] Jensen, 2004[110]	Sikorska, 2004[10]		
tocopherol (vitamin E)				Kyriakidis, 2000[97] Guimet, 2004[122,136] Sikorska, 2004−5[100,101] Zandomeneghi, 2005[99]				
others (iso-α-acids)						Takhar, 1995[111] Tomlinson, 1995[112] Apperson, 2002[113] Sikorska, 2004[10] Christensen, 2005[9]		

of autofluorescence of dietary phorphyrins has been suggested for detection of fecal contamination in meat.[69]

All the described multivariate fluorescence studies of meat used bilinear models to evaluate single-emission spectra. The only multiway study of autofluorescence of meat reported so far was on dry-cured Parma ham, which was monitored throughout processing and aging.[70] A PARAFAC decomposition of the recorded fluorescence landscapes revealed the presence of five fluorophores, of which tryptophan was assigned to be the dominating one. The remaining four components were more difficult to assign; one was suggested to arise from the salting, and two others were related to oxidation products. The PARAFAC component with excitation/emission maxima at 370/470 nm accounted for the second largest fluorescence contribution, and could perhaps be assigned to collagen or NADH in agreement with previous meat studies.

4.2. Fish

Already in 1985 fluorescence measurements were proposed as a method for detecting bones in fish fillets.[71] Autofluorescence of fish was later reported in the form of collagen fluorescence found in cod and salmon using an excitation wavelength of 332 nm.[72] Fluorescence images from this study revealed a significant inner filter effect in the salmon flesh probably caused by strong absorption of the emitted fluorescence light by red pigments such as astaxanthin. Front-face fluorescence has also been suggested for assessment of the freshness of fish.[73] Normalized fluorescence emission spectra using excitation wavelengths of 260 nm (assigned to aromatic amino acids) and 336 nm (assigned to NADH) were evaluated by PCA and suggested to be used for discrimination between different storage times for whiting and mackerel fillets.

PARAFAC has been applied to fluorescence landscapes of fish oil.[74] The PARAFAC decomposition revealed four fluorophores present in the oil samples, of which one was assigned to chlorophyll. The obtained complex fluorescence fingerprints of the fish oils were shown to correlate (indirectly) to the dioxin content in the fish oil, and the method was suggested as a screening method for dioxin contamination.

4.3. Dairy Products

Fluorescence studies of dairy products reported in the literature are dominated by fluorescence assigned to tryptophan, vitamin A, and riboflavin, but fluorescent oxidation and process-derived products have also been described. Recently, chlorophyllic compounds were found in dairy products using fluorescence emission spectra from 400 to 750 nm obtained by illumination with excitation light of 380 nm.[75] Emission peaks between 600 and 700 nm were observed and tentatively validated as chlorophyll *a* and hematoporphyrin.

In several studies of dairy products, fluorescence emission spectra of tryptophan (normalized according to peak area) have been investigated as an indicator of the protein structure in dairy products. Minor shifts in the emission profile evaluated with multivariate data analysis have been related to different locations and the environment of the tryptophan residues in the protein. Front-face fluorescence emission spectra upon excitation at 290 nm were correlated to sensory texture and used for discrimination of the cheese type.[76] A similar approach was applied to study molecular interactions during milk coagulation.[77,78] Several different coagulation systems were studied, and the fluorescence approach including multivariate evaluation allowed the investigation of the network structure and molecular interactions. Within the area of cheese ripening[79,80] a similar approach was used for the study of soft and semihard cheeses; the studies concluded that fluorescence spectroscopy is suitable to provide relevant information related to the cheese protein structure, which was used to discriminate each ripening stage. In addition, selected spectral characteristics of ripened cheeses linked to the initial chemical composition and the initial protein network structure were detected at the early stage of ripening. Front-face fluorescence spectroscopy was also suggested as a rapid method for screening of process cheese functionality;[81] in the presented study functionality was represented by the meltability as measured by dynamic stress rheometry. Application of classification methods on fluorescence spectra recorded on Emmental cheeses[82,83] from different European geographic origins was shown to give correct classification results for approximately 75% of the samples in the first mentioned study and around 45% in the latter.

In a number of studies of dairy products, vitamin A fluorescence has been recorded using excitation spectra with emission at 410 nm. However, no chemical validation (i.e., pure substance measurements) of the assignment of the fluorescence signal has been made. The emission wavelength used seems rather low compared with the fluorescence profile of pure solutions of vitamin A[84] with an emission maximum at 480 nm according to Table 1. Nevertheless, the assignment is not questioned in the papers, and the fluorescence signal has been related to phase transition of triglycerides in cheese.[85] While incorrect assignment of fluorescence may not be crucial when seeking indirect correlations to quality parameters such as rheological characteristics and classifications, it may prevent further interpretation of the chemical system under investigation. A combination of the proposed vitamin A fluorescence and tryptophan fluorescence has been applied in several studies of cheese. The common fluorescence signal was found to correlate with the cheese type as well as with the structure of soft cheese,[86] the rheological characteristics of various cheeses,[80,87−89] and classification of cheese and milk according to origin.[90−92]

Rapid fluorometric methods have been investigated for estimation of the heat treatment of milk based on the intrinsic fluorescence of milk. A combination of fluorescence assigned to tryptophan (emission spectra using the excitation wavelength at 295 nm) and vitamin A (excitation spectra recording emission at 410 nm) was applied in a front-face fluorescence study of milk.[37] Classifications based on PCA of the fluorescence spectra clearly separated the milk samples according to heating and homogenization. Another study used the relation between the excitation/emission peaks at 290/340 and 350/440 nm, assigned to fluorescence from tryptophan and advanced Maillard products, respectively, for a classification according to heat treatment based on measurements of water-soluble milk fractions.[38] The same method was shown to correlate with lysine degradation in milk during heating, although with an adjustment of the peak selection to 330/420 nm for the proposed fluorescent Maillard products.[93] Front-face fluorescence emission has also been used for monitoring intact milk samples using excitation wavelengths of 250 and 360 nm assigned to fluorescence of aromatic amino acids and NADH/FADH, respectively.[39] The

suggested NADH/FADH fluorescence was shown to correlate with heat treatment indicators using principal component regression models.

Riboflavin is considered to be a marker of photooxidation in dairy products, and autofluorescence assigned to riboflavin has been used to describe light-induced changes in dairy products. Front-face fluorescence emission spectra have been recorded from Jarlsberg cheese, sour cream, and cream cheese upon excitation at 380 nm.[35] The fluorescence spectra revealed a significant reduction in fluorescence intensity at 525 nm and a corresponding increase around 415−490 nm upon the expected light-induced oxidation of the samples. The result was ascribed to photodegradation of riboflavin, leading to a fluorescent product. Oxidation of Havarti cheese has also been monitored on the basis of fluorescence excitation/emission peaks at 370/530 and 430/530 nm, ascribed to riboflavin.[94] Autofluorescence in analysis of yogurt confirmed the previous findings by relating storage conditions to the fluorescence signal obtained for emission around 530 nm.[36] Furthermore, a high direct correlation to riboflavin content ($r^2 = 0.98$) was found, verifying the dependency.

Multiway studies of autofluorescence landscapes of dairy products are so far described in two studies,[34,95] where PARAFAC was applied to evaluate the fluorescence landscapes of processed cheese and yogurt, respectively, as a function of storage. In the study of cheeses, fluorescence landscapes with excitation wavelengths from 240 to 360 nm and emission wavelengths of 275−475 nm were recorded. The fluorescence landscapes of the cheese samples were decomposed into four different PARAFAC components assigned to tryptophan, vitamin A, and a nonidentified oxidation product. Two of the resolved PARAFAC components yielded only slightly different excitation profiles, but they both resembled the fluorescence properties of tryptophan with very similar emission loadings with maxima at 347 and 339 nm. Both components were assigned to tryptophan, representing two different populations of tryptophan residues. In general, only the emission profile and not the excitation maxima should be affected by different local environments according to fluorescence theory. However, inner filter effects, which can alter the observed excitation profile and the poor resolution of the excitation wavelength in the study, can justify the assignment. An alternative explanation could be that it was tyrosine residues that were responsible for the absorption in the PARAFAC component with the lowest excitation maximum and that the energy was subsequently transferred to tryptophan by resonance energy transfer.[4] In the latter case, the second PARAFAC component should correctly have been assigned to tyrosine.

4.4. Edible Oils

Frying oil deterioration has been measured with fluorescence emission spectra using five selected excitation wavelengths from 395 to 530 nm and evaluated with multivariate data analysis. The results showed high correlations with quality parameters describing the deterioration.[96] In another study, fluorescence emission spectra from several common vegetable oils were obtained upon illumination by 360 nm.[97] The fluorescence signal was (partly) assigned to tocopherol and chlorophyllic compounds, despite the fact that the proposed tocopherol fluorescence emission at 525 nm certainly does not match the fluorescence properties of pure α-tocopherol.[84]

In olive oil, autofluorescence has been investigated for determination of chlorophylls and pheophytins.[98] Partial least-squares regression models were applied on single-excitation, single-emission, and synchronous spectra, which were shown to be highly correlated to the content of the different pigments ($r^2 > 0.99$). Excitation spectra obtained for emission at 662 nm were found to be the optimal for the data at hand, which makes sense compared to the fluorescence properties of chlorophyll *a* listed in Table 1. Further exploration of the autofluorescence of olive oils showed that the obtained fluorescence signals could likely be assigned to tocopherol, polyphenols, riboflavin, and chlorophyllic compounds. This was concluded in a study also comparing front-face and right-angle sampling geometry.[99] It was suggested that fluorescence spectra obtained with the traditional setup (right-angle fluorescence) contain considerable artifacts and deformations due to self-absorption phenomena, even if the spectra are corrected for inner filter effects, while front-face fluorescence spectra were much less affected by self-absorption.

Recently, studies have been published characterizing complete autofluorescence landscapes with excitation wavelengths of 250−450 nm and emission recorded up to 700 nm of a wide range of edible oils. Fluorescence from various diluted and undiluted oils was investigated, and the obtained signals were assigned to tocopherol and pigments of the chlorophyll group.[100] Furthermore, fluorescence signals were suggested to originate from polyphenols, and fluorescence appearing with excitation around 350 nm and emission between 400 and 500 nm was shown to arise from thermal oxidation. Inner filter effects were clearly evident in the undiluted oils, as seen by the fact that the tocopherol was hardly detectable in the neat oils as opposed to the diluted oils, whereas the fluorescence signals from the polyphenolic and thermally induced compounds were considerably diminished upon dilution. Similar fluorescence landscapes have also been used for classification of edible oils.[101] The landscape approach was compared to synchronous scanning fluorescence spectroscopy to characterize and differentiate edible oils, including soybean, sunflower, rapeseed, peanut, olive, grape seed, linseed, and corn oils. Both methods provided good discrimination between the oil classes with a low classification error based on the nonparametrical *k* nearest-neighbors method and linear discriminant analysis.

Guimet et al. in 2004[102] applied PARAFAC to evaluate complete fluorescence landscapes of olive oils. The decomposition of the olive oil fluorescence revealed the presence of four different fluorophores, of which the major one was assigned to chlorophyll. Two of the derived fluorophores were assumed to be oxidation products with excitation maxima around 350 nm in agreement with previous findings and verified by the fact that they were practically absent in the virgin olive oils, as opposed to the refined oils. The last PARAFAC component in the study with an excitation/emission maximum around 350/525 nm was erroneously assigned to tocopherol with reference to the earlier study of olive oils.[97] However, as previously mentioned, this component does not agree with the fluorescence characteristics of tocopherol found in the literature.[84]

4.5. Cereals

Since the early 1980s fluorescence spectroscopy in combination with PLS regression was used for prediction and classification of botanical tissue components of complex wheat flour[17] and rye flour[103] samples. The classification

was based on excitation at 275, 350, and 450 nm, yielding fluorescence emission maxima at 335, 420, and 520 nm, respectively. The fluorescence was assigned to aromatic amino acids (excitation 275 nm) and ferulic acid (excitation 350 nm). The fluorescence peak at 450/520 nm was not originally assigned, but probably originates from riboflavin, as later verified in a study of wheat flour fluorescence, where a standard addition technique was used for validation of this assignment.[104] The ferulic acid and riboflavin fluorescence was later applied in several studies monitoring wheat flour refinement and milling efficiency using fluorescence imaging,[105−108] and autofluorescence was recently suggested for classification of wheat cultivars.[109] Zandomeneghi in 1999[40] investigated the intrinsic fluorescence of cereal flour more thoroughly and optimized the conditions for recording front-face fluorescence from intact samples. He found three major fluorescence peaks present in the flour samples similar to the ones previously described; one was assigned to amino acids and another one to riboflavin.[104] The signals previously assigned to ferulic acid were not assigned in this study, but suggested to originate from vitamin E or B_6. Fluorescence landscapes of oatmeal samples have also been recorded in an attempt to monitor oxidative changes.[110] The obtained fluorescence signals were comparable to the findings in flour samples and assigned to tryptophan, riboflavin, and an unidentified oxidation product. Is was also shown that the fluorescence signals could be correlated to the hexanal concentration in oatmeal and that during storage time the signals decreased especially in the excitation wavelength range around 450 nm.

4.6. Beer

In 1995 a patent suggested using autofluorescence as a novel and rapid method for monitoring the bitterness in beer,[111] and the approach was pursued in a recent study.[9] Bitterness in beer is primarily determined by the amount of iso-α-acids, which originate from the hops. Fluorescence was suggested for quantification of these (presumably) fluorescent bitter acids, which normally appear in concentrations around 10−40 ppm in beer. An alternative approach toward bitterness determination in beer using europium-induced delayed fluorescence to detect the amount of iso-α-acids in beer was later proposed by Tomlinson et al. in 1995.[112] This technique requires a sample preparation, namely, addition of europium, to separate it from the background fluorescence through a gate time delay. The sample preparation step makes the approach more cumbersome compared to measuring autofluorescence of intact beer, yet the method is much faster than the traditional bitterness determination of beer that involves an extraction step. The topic is of commercial interest, since the traditional method today is carried out as a routine quality control analysis in all modern breweries.

A more thorough description of the intrinsic fluorescence in beer was reported by Apperson et al. in 2002,[113] suggesting that the complex fluorescence characteristics of beer arise from amino acids, complex polyphenols, and iso-α-acids. Similar fluorescence was observed in another study;[9] however, the fluorescence contribution from the bitter acids did not appear obvious upon inspection of the signals. In a recent study of fluorescence landscapes of beer,[10] classification of eight individual beers was evaluated on the basis of their intrinsic fluorescence characteristics. The classification was performed by measuring each beer 12 times, and by the use of the nonparametrical *k* nearest-neighbors method and linear discriminant analysis, it was possible to discriminate between the replicate measurements. The paper did not show if it was possible to group different categories of beers, e.g., light, dark, ale, and lager. The obtained fluorescence signals from the beer were suggested to originate from aromatic amino acids, NADH, riboflavin, and vitamin B_6.

4.7. Fruit and Vegetables

For several decades chlorophyll fluorescence has been considered an intrinsic probe for the photosynthesis in plants.[114,115] Thus, chlorophyll fluorescence has been suggested as a tool for evaluating the heat tolerance of tropical fruits,[116] changes in apples during maturation, ripening, and senescence,[117] the quality of apple juice during processing,[118] and the ripening of papaya fruit,[119] among many others. The mentioned studies all evaluate the fluorescence measurements by the calculation of various fluorescence indices or ratios and by univariate comparison of these to the quality parameters. However, few studies have undertaken a multivariate approach in, for example, classification of apples with fluorescence imaging[120] and prediction of three levels of mealiness in apples using fluorescence kinetics and different mathematical classifiers.[121] In the first of these studies an apple classification method that employed a hierarchy of two neural networks was developed. The system reached 95% accuracy using a test material classified by an expert for "bad" and "good" apples.

Chlorophyll, which gives rise to fluorescence found in plants, can be considered of special interest as an indicator substance due to the fact that the chlorophyll can be found in food products during processing and in several steps throughout the food chain, as seen from the presence of chlorophyll found in fluorescence studies of dairy products[75] and vegetables[122] as well as fish oil.[74]

The autofluorescence of apple juice has also been explored, on the basis of excitation at 265 and 315 nm.[123] The obtained fluorescence emission spectra were evaluated using the PCA and PLS models to classify the juices according to variety and to relate the measurements to the maturity of the apples.

4.8. Sugar

Several examples of the application of fluorescence and chemometrics in the analysis of sugar have been published within the past decade, and the application area has served as a pioneering platform for applying multiway models to autofluorescence landscapes.[124] Fluorescence analyses of sugar and sugar solutions are all based on measuring the impurities in the sugar, since sucrose itself does not possess the ability to fluoresce. The foundation for applying fluorescence in the analysis of sugar was discovered many years ago, as the purity of sugar samples already in the 1940s was evaluated on the basis of an inspection of the fluorescence arising upon illumination with ultraviolet light.[125] This kind of quality control was further investigated and approached more scientifically in 1995 when the fluorescence of crystalline beet sugar and beet sugar juices was recorded and evaluated with chemometrics.[125,126] The fluorescence emission spectra upon excitation of 230, 240, 290, and 330 nm were concatenated and multivariate models used to explore the data. The fluorescence signals dominated by the aromatic amino acids were used for classification of the sugars according to the production site and for prediction of several

quality and process parameters. The findings were later verified on sugar solutions[1] and sugar crystals.[127]

Front-face fluorescence spectroscopy was also recently proposed for authentication analysis of the botanical origin of honey.[128] Fluorescence emission spectra for excitations at 250, 290, and 373 nm were in combination with excitation spectra for emission at 450 nm recorded and evaluated using PCA scores for a linear discriminant analysis classifying the honeys according to floral origin.

More thorough investigations of impurities in various sugar juices were carried out when multiway chemometrics was applied in the evaluation of fluorescence landscapes of sugar.[1,129] PARAFAC revealed four fluorophores that were responsible for the main fluorescence of beet sugar solutions. Two of the components were assigned to tyrosine and tryptophan originating from the beet, on the basis of their derived fluorescent properties. PARAFAC was also applied specifically on fluorescence landscapes of raw cane sugar[130] and solid beet sugar.[131] It was shown that classification according to production site and correlation to important sugar quality parameters such as color, ash, and α-amino N was possible by measuring directly on the crystalline samples. The results obtained were comparable to results obtained on water-diluted samples. Further investigations of the underlying fluorescence phenomena in various sugar and sugar juice samples were carried out by comparing the decomposed fluorescence components to a chromatographic separation of sugar solutions.[132,133] The fluorescence was suggested to arise from colorant polymers formed in Maillard reactions during the sugar processing and a polyphenolic compound in addition to the more straightforward assignments of tyrosine and tryptophan.

5. Conclusions and Perspectives

It is unquestionable that the intrinsic fluorescence from intact food systems contains valuable information on the quality and sensory properties of food products. Fluorophores from raw food materials as well as process-induced fluorescent substances have been shown to provide information about the quality of food products, including food authenticity and influence of processing. Our literature survey revealed an increasing amount of research in the field within the past decade facilitated by the widespread use of chemometrics. The increasing research activities can hopefully address some of the challenges of fluorescence measurements of intact food samples and further explore the chemical systems and causality, which in many cases are not fully understood, as indicated by the tentative assignments of fluorophores in several of the application studies.

The focus of this review was on multivariate applications of fluorescence to food analysis. The new developments in especially multiway multivariate data analysis combined with the high sensitivity and the relatively unique 2D measurement conditions of fluorescence spectroscopy makes a unique and powerful combination with a tremendous unexploited potential for on-line process control in the so-called process analytical technology (PAT) concept. For this purpose fluorescence sensors will be able to provide complementary information about the raw materials and the processed food compared to the information provided by the abundant near-infrared (NIR) sensors, which are based on molecular overtone vibrations and thus less sensitive and specific.

Fluorescence is ideally suited for on-line measurements, because it is multidimensional, selective, and sensitive and because industrial on-line fluorescence sensors are highly feasible, as the wavelengths of the visual illumination and the fluorescence signals with practically no loss can be transmitted over long distances using quartz fibers, making several measuring points possible using a single spectrometer. Then why are there practically no on-line applications, except a few examples in batch fermentors? There is no doubt that the ongoing technological progress can deal with the challenges of standardization (the fluorescence signal is not recorded relative to an incident light beam as in absorbance spectroscopy) and configuration of future fluorescence sensors. We believe that the main challenge for a more useful implementation of multivariate autofluorescence in PAT is related to its high sensitivity, i.e., that minute changes in the raw materials can change the signals dramatically and thus will require real-time or floating multivariate recalibration schemes.

Fluorescence has been known for over 300 years and is still gaining momentum in biology, biotechnology, biochemistry, chemistry, and physics, but thus far it has not been extensively used as an on-line sensor in the food industry. With *mathematical chromatography* in the form of multiway analysis of fluorescence landscapes, the two-dimensional nature of fluorescence data is utilized to improve the selectivity of fluorescence analyses and to expand the potential applications to complex chemical systems such as food samples.

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