PEER-REVIEWED ARTICLE

Food Protection Trends, Vol 36, No. 6, p. 412–419

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Effect-based Analytics: New Concepts for Food Control and Safety

ABSTRACT

Food and feed control depends on specific detection of individual substances or groups of chemically-related substances by classic methods of analytic chemistry. This approach implies that effects of unknown contaminants will remain undetected. To compensate for this issue and in order to meet the increasing requirements in the framework of food and feed control, new approaches should be explored, including effect-based analytics. The underlying principle of effect-based analytics is a screening approach that is not tailored to distinct chemical entities but rather designed to detect biological effects of a sample in selected test systems. This approach offers the advantage of also detecting effects of yet unknown substances. Detailed knowledge of the molecular mechanisms accounting for a certain toxicological effect is a prerequisite for the development of suitable test systems. In this paper, the potential of and prospects for effect-based analytics will be illustrated, along with a description of the current status of the implementation of this concept into the system of food inspection and control. Furthermore, challenges in the field of effect-based analytics will be discussed.

INTRODUCTION

Regulation (EC) no. 178/2002 of the European Union specifies that no unsafe food may be placed on the market *(12)*. Food is regarded as safe only if it does not pose health risks to consumers. Thus, the wording of the law centers on the *effects* of the whole food on human health, not on the presence of individual compounds. Potential health hazards might be based on the presence of contaminants and pesticide residues, as well as on natural constituents of the foodstuff. For some of these substances, maximum levels that may not be exceeded in food and feed have been defined.

Current status of analytics in food and feed control

The basic principle of current food and feed control is the detection of individual substances or groups of chemically-

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related compounds by well-developed methods of analytical chemistry. Food safety in the European Union with regard to these chemical substances has therefore gained high standards and a high degree of reliability. As stipulated by law, the respective authorities of the member states carry out a plethora of analyses, of which only a very few give positive results for the presence of banned chemicals or for false or misleading declaration. For example, results from over 9000 analyses of food samples, conducted yearly on the basis of §§ 50 – 52 of the German "Lebens- und Futtermittelgesetzbuch" (food and feed act) *(2)* are available on the website of the Federal Office of Consumer Protection and Food Safety *(8)*. The fraction of unknown contaminants or residues, as well as natural ingredients with potential harmful effects, remains entirely undiscovered when this approach, based on the search for only known individual toxic substances or groups of substances, is used. At present, food and feed control authorities are barely able to perform a time-consuming and costly identification and analysis of yet unknown substances, because of constraints of financial and human resources. In principle, food and feed control is possible only at the level of random testing.

The principle of effect-based analytics

To meet the increasing requirements of food control laboratories, authorities should tread new paths in food and feed control and safety research. Techniques that permit the simultaneous detection of many different chemicals and/or are suited for the identification of "suspicious" samples in the course of a screening approach, thus allowing for a resource-saving, purposeful use of classic analytical methods, are especially eligible for future use in order to cope with the rising number of samples and analytes. Effect-based analytics is such a novel approach. Its basic principle is to identify biological effects of the whole sample, rather than of individual chemical entities, in specific target test systems. Upon detection of biological effects in samples tested with such an approach, subsequent identification and quantification of the specific substance responsible for the observed effect can be performed by means of classic analytical methodologies.

An inherent advantage of biological test systems, including cell cultures, cell components and isolated receptors, is their ability to identify not only well-characterized substances of interest by detection of their biological effect, but also to detect the effects of unknown compounds that elicit similar biological responses in the respective test systems. The goal of effect-based analytics, therefore, is the sensitive and mechanism-of-action-specific detection of a broad chemical spectrum of toxicologically-relevant substances. Effect-based analytics, therefore, constitute a tool that, in principle, is able to discover unknown active components in food and feed undetected by means of classic analytical methods, since the latter focus on the specific detection of selected, known individual substances. In this context, it has to be noted that, historically, effect-based model systems have played a role in food safety in the form of animal experiments such as rodent bioassays used in detection of marine biotoxins *(4)*. These assays, however, are under debate because of ethical issues, problems with high variability and the difficulty of interspecies extrapolation, thus underlining the need for the development of alternatives, either in the form of *in vitro*-based biological test systems or in the form of chemical-analytical methods *(4)*. Long-term *in vivo* studies in rodents, essentially effect-based test systems, are also still important in authorization processes for certain foods, as in the case of mandatory 90-day feeding study in the authorization process for genetically modified food and feed according to regulation (EC) 1829/2003 *(13)*. However, as almost irrelevant for routine food control because of their duration and complexity, such studies will not be further discussed here.

Development of effect-based biological test systems

A substantial prerequisite for the development of bioassays for effect-based analytics is in-depth knowledge of the respective mechanisms of action of substances or substance groups, with a special focus on the interference by the compounds with specific intracellular signaling pathways, i.e., the identification of so-called "adverse outcome pathways" *(5)* or "pathways of toxicity." "Omics" technologies may be the methods of choice for their identification. These methods mainly encompass global alterations and disturbance of mRNA expression (transcriptomics), protein composition (proteomics) or metabolite patterns (metabolomics) induced by a certain substance or by a group of substances in the target cell population. Using data from these investigations, bioinformatics is the methodology of choice for identifying molecular biomarkers, which reliably and reproducibly indicate measurable downstream consequences of addition of the toxic substance to the cells. Such biomarkers will then be integrated into biological test systems.

Comparable goals are followed in the United States (U.S.) in the course of the research project "Tox21", which was initiated in 2009 as a collaboration among the EPA (Environmental Protection Agency), the NIH (National Institute of Health), and the U.S. FDA (Food and Drug Administration) *(17, 18)*. Within the "Tox21" project, novel high-throughput *in vitro* methods are applied in order to identify key molecular events in the toxicity of a plethora of chemicals that have not been characterized previously with respect to their mechanisms of toxicity. Goals of the project are the identification of chemicals that lead to biological responses and to determine their mechanism of action in biological systems, to prioritize compounds for more extensive evaluation of their toxicological properties, to develop novel predictive models for the assessment of potentially hazardous substances and their impact on human

health, and to annotate all toxicologically-relevant human biochemical pathways and design tests that can measure the response of these pathways to chemicals. Thus the position presented in this paper is directly related to the aims and outcome of the "Tox21" project. Results from this project might be picked up by the respective food safety authorities and integrated into food control processes and related research projects. For more detailed information on this project, please refer to previously published literature *(17, 18)*.

Application of effect-based analytics in food and feed control

Effect-based analytics is still only sparsely used in food and feed control. For a list of examples of effect-based methods and their relevance for food control see *(Table 1)*. The reasons for the sparse use of effect-based approaches are complex; they include a lack of appropriately-validated methods, and perhaps the absence of a legal basis for application of the methods, insufficient knowledge of cell cultivation methods, and perhaps the nonexistence of necessary equipment for cell culture experiments in food and feed control laboratories. In addition, establishment of such methods in a laboratory requires complex and expensive internal validation procedures. Moreover, clear legal regulations on the consequences of positive results are still missing.

Two different approaches need to be distinguished for the application of effect-based analytics in the field of food safety:

- "Non-target analytics": In this approach, a certain biological effect is detected as the sum parameter of

Table 1. Selected effect-based test systems, relevance, and detected toxins

Test name	Principle	Relevance	Reference(s)	Substance examples
CALUX and related assays	In vitro luciferase reporter assay for AHR activation	- Formally validated assay - Routine use in some food control laboratories	*(3, 11, 19)*	Dibenzo-dioxins, PCBs, polycyclic aromatic hydrocarbons
Cytotoxicity testing	Different in vitro metabolic activity assays	- Not in routine use	*(20, 21)*	Many different
EROD	In vitro activity assay for AHR-induced metabolic enzyme	- Occasionally used in food safety	*(10)*	Dibenzo-dioxins, PCBs, polycyclic aromatic hydrocarbons
Estrogen receptor binding assays	Competitive in vitro binding to ER	- Formally validated assay - OECD guideline available - Occasionally used in food safety	*(16)*	Steroid hormones, diethylstilbestrol, genistein, bisphenol A
LUMI-Cell ER and related assays	In vitro luciferase reporter assay for ER activation	- Formally validated assay - OECD guideline available - Occasionally used in food safety	*(9, 15)*	Steroid hormones, diethylstilbestrol, genistein, bisphenol A
Mouse/rat bioassay	Acute lethality to rodents after food extract injection	- Standard method according to regulation (EC) no. 2074/2005 until end of 2014 - Currently only for periodic monitoring for detecting unknown marine toxins	*(4, 14)*	Marine biotoxins, e.g., ciguatoxins, domoic acid, saxitoxin, yessotoxins

different possible molecular modes of action converging at the respective biological effect. For example, cytotoxicity or genotoxicity can function as endpoints of such analyses. This concept implies that neither specific information about the identity of the substance(s) that are causally responsible for the observed effect, nor direct evidence for the underlying molecular pathway(s) of toxicity, is retrieved.

- "Targeted analytics": By contrast, the "targeted analytics" approach is focused on the detection of a known specific downstream effect of one or several substances. For example, the selective detection of dibenzo-dioxins and -furans or similarly-acting compounds by a test system specifically adapted to the particular mode of action, e.g., in the form of a reporter gene assay, constitutes a potential cost-effective screening method. The most prominent representative of this type of assay in effect-based analytics is the CALUX (chemical-activated luciferase gene expression) assay *(11)*, which will be presented in more detail in the next section.

The molecular mode of action needs to be known when a "targeted analytics"-type assay is to be developed. This mode of action has to be specifically measurable via a respective biological or biochemical endpoint at sufficient sensitivity. As to be detailed, this approach has been used successfully for the detection of dibenzo-dioxins, in combination with sample pre-processing methodology, based on physicochemical principles.

The "non-target analytics" approach is aimed at the sole identification of the biological effect of a substance or sample and may be used in the future within the framework of safety assessment of food and feed. It is, however, not suited for use as a stand-alone approach for food and feed control in its present form, since the chemical-analytical identification of an individual causative substance is still required by law. Nonetheless, "non-target analytics" allows for the detection of a broad spectrum of toxicologically-relevant compounds by assessing their biological effects. In case of positive results, the causative agents need to be identified subsequently by means of classic methods of analytic chemistry *(Fig. 1)*. The latter task might be, on a case-by-case basis, very complex because of a lack of appropriate field-tested strategies and experience. Further research in this field is necessary.

The CALUX assay as an example of successful application of effect-based analytics

As the only current application of effect-based analytics in food control in Germany, the substance-oriented CALUX assay *(6, 11)* for the detection of dibenzo-dioxins and similarly-acting compounds is performed in a rather small number of laboratories. This test system will be presented in more detail as a prototype assay for test systems based on a luciferase reporter gene system. For additional information about details and applicability of the assay, please refer to published literature *(3, 11, 19)*. The latter screening method has recently been implemented in the European Union by the setting of new criteria for the application of this bioanalytical screening tool (Commission regulations (EC) No. 252/2012 and 278/2012). The CALUX assay is used most frequently as a screening tool for dibenzo-dioxins, while other test systems such as the micro-EROD (ethoxyresorufin-o-deethylase) bioassay *(10)* are also in use. The common basis of these assays is the specific binding of dibenzo-dioxins to the aryl hydrocarbon receptor, AHR, sometimes also termed dioxin receptor *(1)*. For the CALUX assay, mouse or rat hepatoma cell lines were stably transfected with a DNA construct containing a reporter gene, in this case firefly luciferase, under the transcriptional control of specific AHR-responsive DNA sequences, the so-called dioxin response elements. If dibenzo-dioxins or comparable AHR-agonistic substances are present in the sample of interest, these substances will bind to the AHR as ligands, triggering binding of the activated receptor to its responsive DNA sequences. This step is followed by transcription of the downstream firefly luciferase reporter gene and by translation of the resulting mRNA into protein. The cellular amount of this protein can be determined via the metabolism of a specific firefly luciferase substrate whose chemical conversion by the enzyme is linked to emission of light. The CALUX assay is used by single laboratories in the European Union for routine analysis of food, feed and environment samples for their content of polychlorinated dibenzo-dioxins and -furans (PCDD/PCDF), as well as polychlorinated biphenyls (PCB).

According to the European Reference Laboratory (EU-RL) for dioxins and PCB in feed and food (Freiburg, Germany), this test is, in principle, well suited for the pre-selection of suspect samples *(15)*. It can be assumed that high-throughput laboratories are capable of screening approximately 2,500 food or feed samples per year using this technique. The implementation of the CALUX assay, however, requires remarkable efforts with respect to sample preparation, internal validation and routine quality control procedures. Once established and properly conducted, the method is valid with regard to positive/negative decisions and is comparably quick and cost effective to perform.

Such a screening method facilitates a higher sample throughput, thus enabling food and feed control laboratories to identify suspect samples more easily and quickly. For example, as reported in the course of a workshop on effect-based analytics held at the German Federal Institute for Risk Assessment (BfR) in October 2012 in Berlin *(7)*, the RIKILT (Rijks Kwaliteitsinstituut voor Land-en Tuinbouwproducten) Institute of Food Safety, Wageningen, The Netherlands, classified approximately 90 percent of the samples analyzed in the course of a "dioxin crisis" in 2011 as negative via pre-selection with the CALUX assay, which meant that only the remaining 10 percent of the

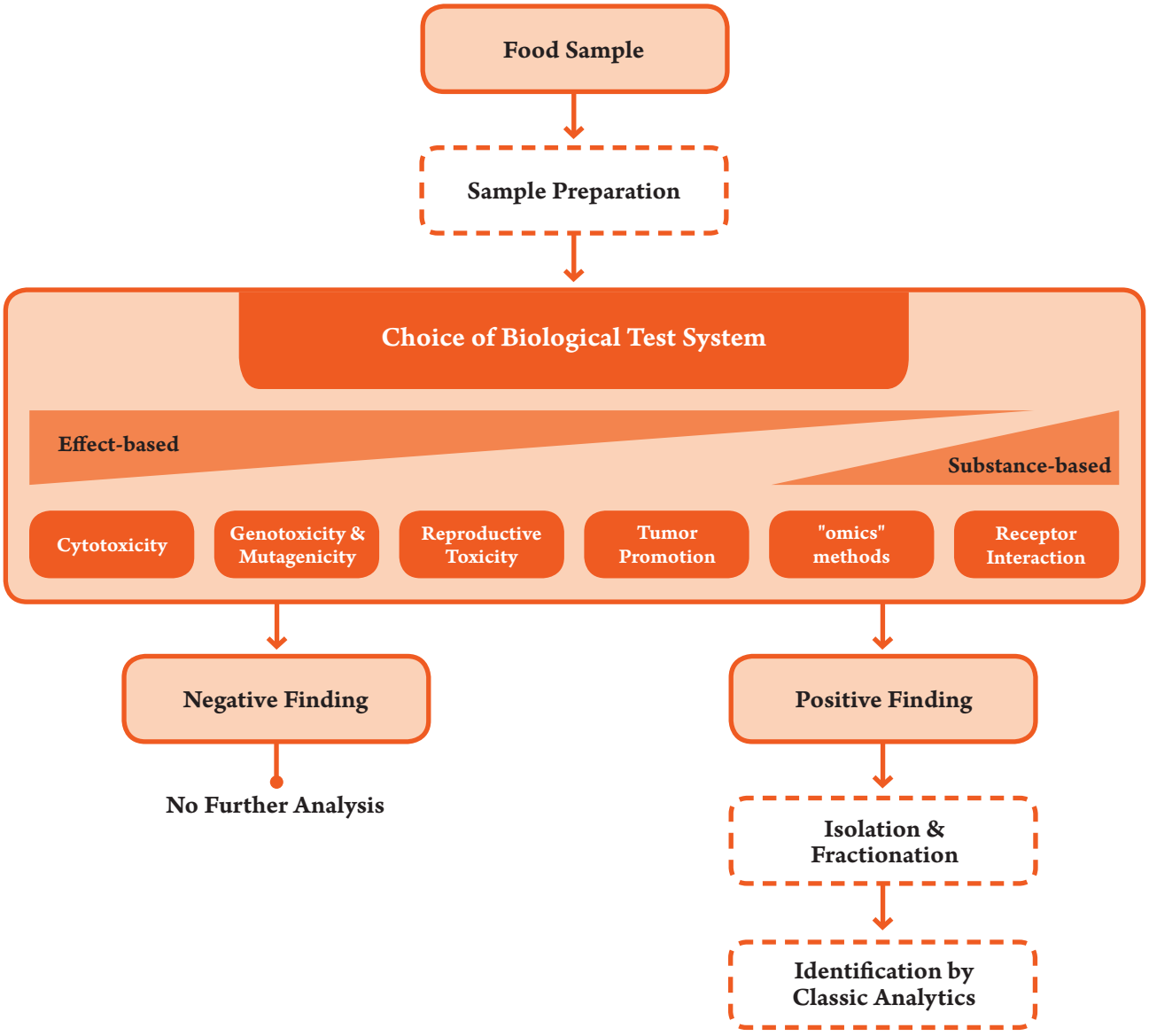

Figure 1. Biological test systems for application in effect-based analytics. Test systems might be combined, depending on the endpoints that need to be addressed. Positive findings have to be verified by subsequent specific methods of classic analytical chemistry.

samples had to be analyzed using the classic methodology of GC/HRMS (gas chromatography/high resolution mass spectrometry). Using the CALUX assay, the RIKILT Institute was able to detect dioxins in eggs from hens that had been fed contaminated maize meal from the Ukraine in May 2010.

The German Federal Ministry of Food and Agriculture commissioned the Federal Institute for Risk Assessment (BfR), in the course of a dioxin contamination incident in 2011, to evaluate the performance of the CALUX assay for the analysis of dioxin contamination in food and feed in close collaboration with the EU-RL for dioxins and PCB in feed and food.

The investigators reached the following conclusion: Cell-based bioassays are generally suitable as screening methods for the pre-selection of "positive" samples, according to the commission regulations (EC) no. 1883/2006 and no. 152/2009. The CALUX assay is such a method. The concentrations of dioxins in the positive samples, however, have to be subsequently verified using other techniques, as laid down in regulations (EC) no. 1883/2006 and no. 152/2009, in order to determine whether the contained amounts of the contaminant do in fact exceed the legal maximum levels. The performance of the bioassay depends on the proper execution of the test

according to the scientific state of the art and considering aspects of quality management.

On the basis of available data, the BfR concluded that the RIKILT Institute correctly applies the CALUX assay according to the legal specifications, as long as the results are presented in the form of "positive"/"negative" decisions.

Results clearly show that extensive validation and standardization is required prior to routine application of the CALUX assay or other comparably constructed reporter gene assays for the detection of other specific effects. The validation and standardization of the CALUX assay done by the EU-RL for dioxins and PCB in feed and food are path-breaking in this field. The BfR supports these activities because the application of screening methods allows for an increased control density and thereby improves the safety of food and feed. As a next step, a Europe-wide ring trial might be conducted, possibly organized by the EU-RL and involving the participation of the national reference laboratories in the European Union member countries. Such a ring trial might increase the overall acceptance of the bioassay in the European Union, if the performance of the bioassay, compared to classic analytical dioxin determination methods, is demonstrated sufficiently.

Effect-based bioassays for hormonal activities

The CALUX method for the detection of dibenzo-dioxins and PCB, as already detailed, definitely has the highest potential to find its way into routine food control in a medium-term perspective. Further promising candidates are similar bioassays aimed at detecting estrogenic or androgenic effects. These test systems are based on the activation of the estrogen receptor (ERα) or the androgen receptor (AR), respectively, and detect hormonally-active compounds, i.e., potential endocrine disruptors, by means of their cellular downstream effects. Validated OECD (Organization for Economic Co-operation and Development) guidelines for the detection of ERα-dependent effects have become available *(15, 16)*. Like the CALUX assay, these test systems are very sensitive and allow for detection of specific agonists in a nanomolar, or potentially picomolar, range. However, they are less selective and detect a broader range of substances that share the ability to activate the receptors ERα or AR, respectively. Thus, the latter assays will most likely be suited only for some special applications, e.g., detection of hormonally-active compounds in matrices, such as drinking water, that do not naturally contain such chemical entities. Despite the fact that these assays do not provide data on the exact nature of the causative substances, they allow prediction of the overall hormonal activity of a sample under investigation, thereby perhaps constituting a meaningful complementary method to the classic analytical determination of endocrine disruptors and hormones. A study of the RIKILT Institute, presented during the BfR symposium 'Effect-based analytics in food control' in October 2012 *(7)*, illustrates the potential of these bioassays: Eighteen dietary supplements were tested by classic LC-MS/MS (liquid chromatography-mass spectrometry) procedures for the presence of 49 different anabolic steroids. As a result, 11 samples were classified "positive" for the presence of anabolic steroids, whereas the other seven samples were "negative." In two of the 7 "negative" samples, a yeast androgen bioassay developed at the RIKILT Institute detected substances with androgenic properties that had not been detected by use of the classic analytical methods.

As detailed already for the CALUX assay, use of bioassays for detection of hormonally-active substances in the surveillance of food and feed requires thorough validation prior to application in an ISO 17025-certified laboratory. As is the case for detection of dioxin-like effects, the issues of interpretation of positive results for hormonally-active substances and possible consequences of such findings for food control need to be clarified. Effect-based analytics for potential endocrine disrupters is also a possible strategy for testing for unauthorized use of anabolic substances in animal feeding. There, newly developed compounds are initially not the focus of classic analytical methods, thus hampering the detection of administration of novel anabolic substances. In addition, classic analytics will not detect a combination of different individual substances that have each been fed below their level of quantification, whereas effect-based analytics might detect the sum of common cellular effects of similarly-acting anabolic compounds.

APPLICATIONS AND FURTHER CHALLENGES

Biological test systems offer the chance to complement classic instrumental analytics with screening procedures and to obtain important information with regard to the safety of food and feed. They enable a more focused use of time-consuming and expensive compound-centred analyses and might liberate resources in food control, which can offer the advantage of increasing the number of analyzed samples and consequently improving consumer protection. With regard to the different types of hazards potentially present in food, the applicability of effect-based analytics will most likely be focused on chemical hazards, rather than on other types of hazards (e.g., pathogens, radiation) that might be detected faster and/or more easily by other methods (e.g., presence of pathogen DNA, physical measurement of radiation).

In terms of liberation of resources, however, it also must be noted that analytical search for an unknown substance, as it currently done after "positive" results are obtained in a screening assay, might turn out to be very time consuming. Thus, before implementing such screening tests in routine analyses, it should be clarified whether the detection of a certain activity in a sample, e.g., estrogen receptor activation, must inevitably be followed by the identification of the causative agent or whether the proof of estrogenic activity alone might be sufficient to trigger consequences and

Table 2. Comparison of effect-based and classic analytical methods

	Effect-based analytics	Chemical analytics
Endpoint	Effect in biological test system	Presence of chemical entity
Application	Only few assays in routine use	Regularly used for most chemical hazards
Scope	All substances with a common biological effect	Single substance or group of chemically closely related compounds
Drawbacks	- Has to be followed by chemical-analytical substance identification according to current legislation - No clear identification of causative chemical	- No detection of unknown hazardous substances - No identification of mixture effects
Advantages	- Identification of effects of yet unknown hazardous substances possible - Identification of mixture effects (i.e., several chemicals which influence a biological endpoint) possible - Screening for effects to prioritize samples for chemical analytics	- Identification of causative chemical possible (mandatory according to current legislation) - Well-established methods for important contaminants available

decisions which, at least in some cases, would make analytical substance-specific identification no longer necessary.

Further challenges in the implementation of the concept and of specific methods of effect-based analytics in the control of food and feed are:

- Future development, validation and standardization of bioassays;
- Investigations that allow for the definition of threshold values for certain effects;
- Establishment of a catalogue of criteria for the interpretation of experimental results;
- Estimation of the effort for chemical analytics that would be necessary for the follow-up of positive results from effect-based biological screening tests;
- Building up an infrastructure of cell and molecular biology laboratories at the regulatory authorities and training of staff in order to generate expertise in the field.

All in all, there is still a substantial need for further research on different aspects of effect-based analytics. Additional molecular markers that are demonstrably specific for certain groups of chemicals need to be identified. An optimization of the individual steps of the testing procedures also is required. This approach is especially true for the sample preparation step for routine diagnostics. In addition, regulatory activities aimed at improving acceptance and establishment of effect-based analytics as standard methodology in the field of food surveillance are needed. A summarizing comparison of effect-based testing strategies and classic analytics is presented in *Table 2* in order to provide an overview of advantages and drawbacks of the different methods. Combination of both analytical and effect-based approaches, for example, by using effect-based analytics as a tool for sample prioritization for classic analytics, or by using analytical techniques for pre-fractionation of complex samples prior to effect-based tests, might provide additional perspectives of food safety.

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