EXTERNAL SCIENTIFIC REPORT

Analysis of needs in post-market monitoring of food additives and preparatory work for future projects in this field[1]

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements (Geel)

ABSTRACT

Collection of data on food and feed safety is one of the main tasks of the European Food Safety Authority (EFSA). In the domain of food additives, Member States are performing analyses, but a common survey plan and harmonised data collection are still not in place. The support of the Joint Research Centre (JRC), one of the Directorates General of the European Commission, was requested by EFSA to investigate the availability of methods used for analysis of food additives with highest priority in the most relevant food matrices; to identify the gaps and areas for improvement. JRC was also asked to draft a document with the technical details to be included in a possible future call for ad-hoc projects to address the identified gaps in current MS post-market monitoring activities. In this report the conclusions about the feasibility of the available methods for analysis of most relevant group food additives (colouring substances and sweeteners), based on their performance characteristics are summarised and potential gaps are identified. A MS Excel database is established with the methods and their corresponding scope and method performance characteristics. The method database is linked to the relevant food additive/food category combination.

KEY WORDS

food additives, sweeteners, food colours, analytical methods, performance characteristics

DISCLAIMER

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Any enquiries related to this output should be addressed to dcm@efsa.europa.eu

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SUMMARY

For performing risk assessment, the European Food Safety Authority (EFSA) needs reliable information on the occurrence of food additives in different food categories. As a background for ensuring reliability, the availability of validated methods with required method performance characteristics applied for the determination of the levels of food additives in respective food commodities is essential.

The support of the Joint Research Centre (JRC), one of the Directorates General of the European Commission, was requested by EFSA to investigate the availability of methods used for analysis of food additives with highest priority in the most relevant food matrices; to identify the gaps and area for improvement.

This report gives an overview of the state of art of the methods for the analysis of selected food additives in different foodstuffs, and provides information on method performance characteristics of these methods, as far as this information was available.

The selection of prioritised food additives was based on the analysis of the RASFF database for the period 2010-2012 together with the information on post-market monitoring of food additives in Europe for 2011 collected by EFSA from their network members. Based on this investigation sweeteners and food colours were identified as being of highest priority. A priority list of food additives/matrix combinations with maximum permitted levels (ML) different from quantum satis was compiled based on requirements laid down in Commission Regulation (EU) No 1129/2011, amending Regulation (EC) No 1333/2008.

Databases of ISO, CEN, AOAC, Codex Alimentarius and Member States National Standardisation Institutes were searched for standard methods of analysis for sweeteners and colourants (i.e. the coulouring principles in food colours). The SCOPUS literature database was searched for analysis methods for the mentioned categories of food additives in food, and a list containing the most relevant articles that were published on these topics in the last decades was set up. Special focus was given to the identification of multi-analyte methods and to methods that allow the determination of the respective food additive at the lowest ML which is specified in legislation for the food additive/food category combination.

For the group of sweeteners, certain gaps were identified, e.g. the lack of well characterised methods for the determination of thaumatin (E 957) and the recently approved steviol glycosides (E 960)in any food commodity.

A serious problem for regulatory compliance assessment is provided by the group of colourants with combined MLs. They need at least the application of a number of different methods in order to cover the compounds that are regulated by the ML. However, there are not sufficient methods at present that would allow covering all substances with the combined ML in all regulated food additive/food-subcategory combinations.

There is room for improvement of analysis methods for the group of natural colourants. One of the major challenges there is the availability of reference standards with known purity for the main colouring principle. Also the isolation of the respective colourants from different complex foodstuffs needs to be tackled.

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BACKGROUND AS PROVIDED BY THE EUROPEAN FOOD SAFETY AUTHORITY

Article 27 of the Regulation (EC) No 1333/2008 of the European Parliament and of the Council on food additives, while prescribing rules on monitoring of food additives intake says:

"Member States shall maintain systems to monitor the consumption and use of food additives on a risk-based approach and report their findings with appropriate frequency to the Commission and the Authority"

and foresees the establishment of a common methodology for the gathering of this information.

In line with this prescription, the EFSA Science Strategy 2012-2016 in section 4 'Strengthen the scientific evidence for risk assessment and risk monitoring', states:

"…Whereas the focus in the occurrence monitoring has been initially on microbiological and chemical contaminants, it is broadening into monitoring of chemicals which are subject to a marketing authorisation, such as plant protection products or food additives. This permits to assess whether the exposure envisaged at the time of marketing authorisation matches with the true exposure, when marketed…"

In the domain of plant protection products a systematic collection and analysis of monitoring data has already been established. A report on pesticide residues is produced annually. Veterinary medicine residues data also follow a similar process. In the domain of food additives this process is far less advanced. Member States are performing analyses, but a common survey plan and harmonised data collection are not in place, even if the tools for collecting data on occurrence of chemical substances are already available (Standard Sample Description, Data collection Framework, Web services for electronic data transmission).

In order to set up an efficient and harmonised data collection on post-marketing monitoring of food additives, an exploratory risk-based analysis of needs in relation to this monitoring is required. This work is aimed at ranking food additives, identifying the major possible sources of concern, and checking for the availability in the Member States of proper analytical methods for monitoring the presence of these substances and of sufficient data of appropriate quality on their actual occurrence levels in food. Furthermore, a draft document with the technical details to be included in a possible future call for ad-hoc projects to address the identified gaps in current MS post-market monitoring activities should be developed.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN FOOD SAFETY AUTHORITY

This contract was awarded by the European Food Safety Authority to:

Contractor: Institute for Reference Materials and Measurements - Joint Research Centre - European Commission

Contract title: Analysis of needs in post-market monitoring of food additives and preparatory work for future projects in this field

Contract number: SLA/EFSA/DCM/2012/01

The objectives of the agreement were to focus on post-market monitoring of food additives and prepare:

a. a list of priority substances to be addressed in monitoring and data analysis;

b. a list of analytical methods for these priority substances including analysis of gaps and possibilities for improvement;

c. a draft document with the technical details to be included in a possible future call for ad-hoc projects to address the identified gaps in current MS post-market monitoring activities

Expected deliverables were:

Database including the additives with highest priority and the methods used for their analysis in the most relevant food matrices; Gaps and areas for improvement have to be identified in the database;	by end of October 2012
Draft document with the technical details to be included in a possible future call for ad-hoc projects to address the identified gaps in current MS post-market monitoring activities. Separate requirements/details shall be addressed for ad-hoc surveys on food additives and analytical method development/improvement.	by end of November 2012
Short final technical report, summarising the work done and highlighting the major findings (at the end of the project).	at the end of the project, not later than 10 December 2012

INTRODUCTION AND OBJECTIVES

INTRODUCTION

Food additives are substances added intentionally to foodstuffs to perform certain technological functions, for example to colour, to sweeten or to help preserve foods.

EFSA requires reliable information on the occurrence of food additives in different food categories for performing reliable risk assessment. Reliability of the data supplied to EFSA is supported by the application of well characterised methods by the data producing laboratory, with method performance characteristics satisfying the respective purpose, as well as suitable quality control measures that demonstrate the proficiency of the laboratory in executing the respective analysis.

Consequently, EFSA launched a project for establishing a method performance database with data of published analytical methods for the determination of the most relevant food additives in regulated food matrices and identifying gaps and areas where improvements are needed.

OBJECTIVES

The main objective of this project were to establish a priority list of food additives and to evaluate published analytical methods for the chemical analysis of the prioritised food additives in food, and to examine gaps and possibilities for further development. Food additives, which may be applied in all authorised food categories *ad quantum satis* shall be excluded from the studies.

APPROACH

A kick-off meeting took place at IRMM (Geel) on 12/07/2012. Details of the project were discussed and agreed upon.

For the execution of the project, several information sources were used for gathering information on analytical methods. ISO, CEN, AOAC, Codex Alimentarius and Member States National Standardisation Institutes' databases were searched for standardised analysis methods. Literature search for non standardised analytical methods was performed via the SCOPUS literature database.

Details of the methodology applied for obtaining the information necessary for the project were in chronological order:

a) Entries in the RASFF database were evaluated for the period 2010 to 2012 as well as the data reported by Member States in 2011 to EFSA for post-market monitoring of additives in Europe for establishing the list of food additives, which the study should target (priority list).

b) For the prioritised food additives, a list of food additives/matrix combinations was extracted from the Commission Regulation (EU) No 1129/2011, amending Regulation (EC) No 1333/2008, for which the maximum permitted level (ML) was different from *quantum satis*.

c) ISO, CEN, AOAC, Codex Alimentarius and Member States National Standardisation Institutes' databases were searched and a list of the existing standard methods for the analysis of sweeteners and colourants in food was drawn. Standard methods for food categories not included in the method validation studies by collaborative trial are marked as “methods

assumed suitable for analysis", when, based on the food composition, it is highly probable that the method could be applied for those matrices.

d) The SCOPUS literature database was searched and a list of the most relevant articles published in the last decade on methods for the determination of sweeteners and colourants in food was compiled. Priority was given to multi-analyte methods and methods that allow the measurement of the respective food additive at the lowest ML specified in legislation for the respective food additive/matrix combination. Methods from articles with almost no validation data are not included in the database.

e) Search terms used in database searches were: "food additive", "sweetener", "artificial sweetener", "food colour", "food colourant", "synthetic food colours", "synthetic food dyes", "natural food colours" and the name of all individual sweetener and colour under the identified scope of the project.

f) Layouts for three Microsoft Excel tables (Appendix 1) were prepared for collecting the required method performance data.

f.1 Table 1 links the categories in legislation with the identified analytical methods. Following the request of EFSA the food categories and sub-categories from Commission Regulation (EU) No 1129/2011 are linked with the respected unique codes provided from the FoodEx 2.0 classification system. The names of the food additives and their E-numbers are linked with the respective parameter code from the catalogue of substances (PARAM) of the Standard Sample Description (SSD). Methods are given in the last two columns. A distinction was made between methods which have the respective analyte/matrix combination within their scope, and for which exhaustive information on method performance data is available, and analysis methods which do not have the respective matrix/analyte combination in their scope, but for which applicability could be assumed due to similarity of matrices with matrices in the scope of the method, or methods for which gaps in the method performance data were identified. The earlier methods are labelled as "Methods accepted for analysis", whereas for the latter applicability can only be assumed. They are labelled as "Methods assumed suitable for analysis". The confidence in these methods is lower than in those in the previous category. However, EFSA might use the provided performance characteristics as guidance for the tender specifications of future projects.

f.2 Table 2 provides the bibliographic reference of the methods

f.3 Table 3 provides performance characteristics of the method for the particular analyte.

These three tables are inter-linked via the method codes. The method code consists of M (for method), first digit for food additive class (1 for sweeteners and 2 for food colours), next digit to distinguish between international standards and methods from literature (1 or 2 respectively), and the last digit provides code for the method. The layouts of the tables were sent to EFSA for approval in the second half of July 2012.

g) The database in the form of the filled Microsoft Excel tables was prepared and sent to EFSA at the end of October. The electronic version of the tables consists of the following Microsoft Excel files:

g.1. additives (sweeteners&colours)+method codes by ML and by food category rev.10.xls. The file contains several sheets:

- "E number-food category by E" represents the Part E of ANNEX II of the Commission Regulation (EU) No 1129/2011 with all authorised food additives and conditions of use in food categories grouped by E-numbers;

- "E number-food category by ML" represents the Part E of ANNEX II of the Commission Regulation (EU) No 1129/2011 with all authorised food additives and conditions of use in food categories sorted in ascending order by maximum levels of use;

- "colours+method codes" links authorised food colourants/food category combinations with analytical methods considered suitable for the determination of the respective colourant in the particular food category. The food colourants were coded in addition to E numbers with SSD 2 parameter codes. FoodEx 2 codes were used to classify the respective food category. Hence the applied coding system allows easy integration of data extracted form legislation into EFSAs' data infrastructure. The information is sorted by E-number in ascending order.

- "sweeteners+method codes" links in analogy to the colourants authorised food sweeteners/food category combinations with analytical methods considered suitable for the determination of the respective sweetener in the particular food category. FoodEx 2 codes were used to classify the respective food category. The information is sorted by E-number in ascending order.

g.2. "sweeteners methods database rev.8 to print.xls" and "colours methods database rev.5 to print.xls" are two files with similar structure, consisting of three data sheets:

- "STD methods" are references to published standardised methods;

- "literature ref." is a list of articles referring to methods for sweeteners/colourants. Articles in Japanese or Chinese, for which only abstracts were available in English language, are highlighted in gray. The characteristics of these methods are given in the next data sheet as far as they were extractable from the papers. However, as the suitability of the highlighted methods could not be fully evaluated, due to the language issue, they were not integrated in the list of suitable/probably suitable analysis methods that were identified for each analyte food category combination.

- "Scope and perform. by method" is a sheet that contains the available method performance characteristics for all the methods, mentioned in the previous two sheets. Again the articles in Japanese or Chinese are coloured in gray. In orange colour are those methods which are not described in details in the article, or for which important method performance characteristics are missing and the methods could not be fully evaluated. The methods integrated in the list of suitable/probably suitable analysis methods that were identified for each analyte food category combination are highlighted in green.

h) Two documents were drafted covering the technical details to be included in a possible future call for ad-hoc projects to address the identified gaps in current MS post-market monitoring activities. The drafts were based on a template for technical specifications provided by EFSA.

One technical specification relates to projects on ad-hoc surveys on food additives whereas the other contains technical details for projects on analytical method development/improvement. The two documents were provided to EFSA together with the present report.

RESULTS

1. Establishing a list of priority food additives to be addressed in monitoring and data analysis

The target group food additives for further investigation was determined based on the results from the RASFF portal and on the post-market monitoring data collected from MS by EFSA for 2011.

A search in the RASFF database for notifications in the period 01/01/2010 until 10/10/2012 by hazard category "food additives and flavouring" showed about 400 different types of notifications related to food additives (Figure 1). 130 of them (32 %) concerned the groups of food colours and sweeteners, 110 (27 %) were related with undeclared, unauthorized or too high content of sulphites in different foods and 88 (22 %) were related to preservatives as benzoic, sorbic, propionic and citric acids and their salts.

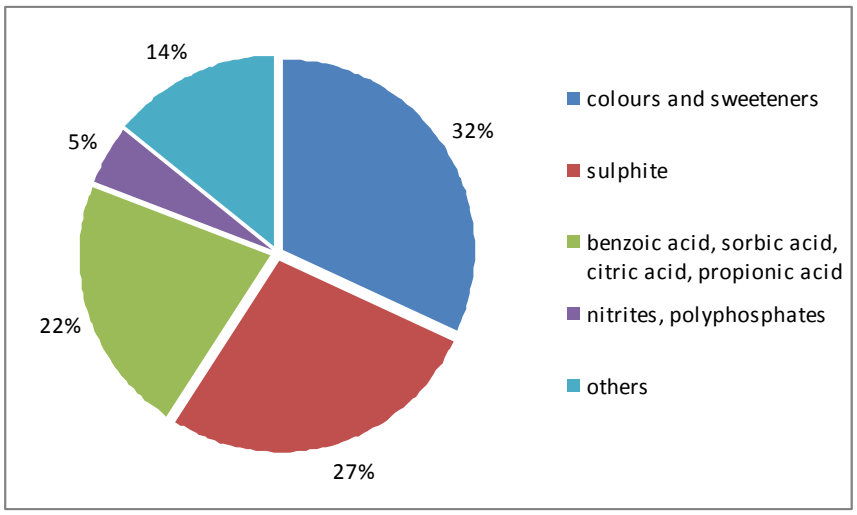

Figure 1: Number of notifications in RASFF system for period 2010-2012.

A summary overview of the post market monitoring data for food additives collected for 2011 in Europe was provided by the EU MSs to EFSA. It shows (Figure 2) that about 165 000 analyses were performed, of which 28,1 % are related to food colours, 20.8 % concerned artificial sweeteners, and 24.2 % - benzoates and sorbates, and 5.7 % to sulphites. This data indicates the importance the MS attribute to sweeteners and food colours.

Apart from sweeteners and food colours, sulphites are other groups of food additives with high percentage of RASFF notifications for 2010-2012. They are chemical agents added to food, including beer and wine that prevent bacterial growth. The term "sulphites" includes sulphur dioxide and the salts formed from sulphurous acid, such as sodium sulphite or potassium metabisulphite.

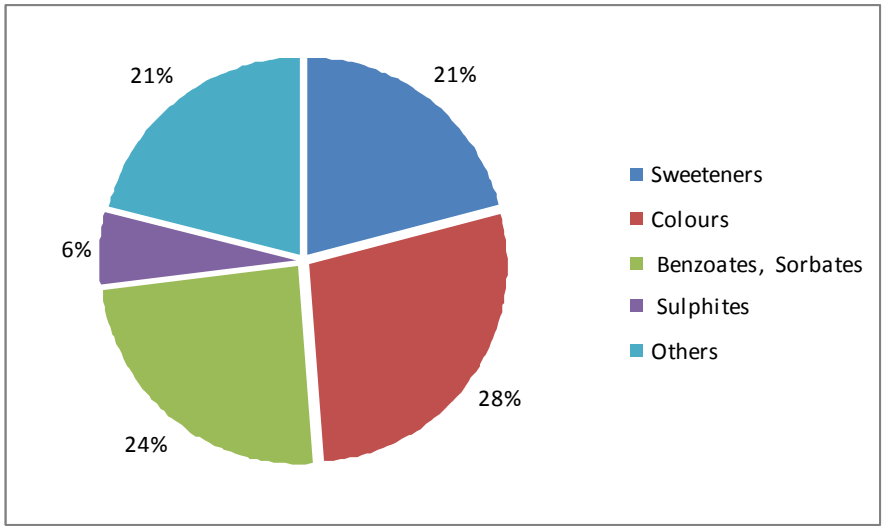

Figure 2: Post-market monitoring data for food additives, collected by EFSA for 2011.

Seventy five food sub-categories with MLs for sulphite (including 32 subcategories only for fruit and vegetables) were extracted from Commission Regulation (EU) No 1129/2011. Maximum levels are expressed as SO_2. They relate to the total quantity of SO_2 stemming from all sources. However, a SO_2 content of below 10 mg/kg or 10 mg/l is considered absent.

Two European and one ISO Standards are available for the determination of SO_2, covering a wide range of foodstuff. Hence the determination of sulphites is not considered a potential analytical problem.

The Standardised methods are:

EN 1988-1:1998 Foodstuffs - Determination of sulphite- Part 1: Optimized Monier-Williams method

EN 1988-2:1998 Foodstuffs - Determination of sulphite- Part 2: Enzymatic method

ISO 5522:1981 - Fruits, vegetables and derived products - Determination of total sulphur dioxide content

Notifications on benzoic and sorbic acid are as well often listed in the RASFF database. They are also frequently analyzed (22% of all analysis) by MS. ISO methods are available for different food matrices and their analytical determination should not present a big challenge to the control laboratories

ISO 22855:2008 Fruit and vegetable products - Determination of benzoic acid and sorbic acid concentrations - High performance liquid chromatography method

ISO 9231:2008 Milk And Milk Products - Determination Of Benzoic Acid And Sorbic Acid Contents

Based on the above mentioned data the conclusion was drawn that the most sensitive and complex groups of food additives, being most monitored and having greatest number of notification trough

RASFF are sweeteners and food colours. As a consequence they were chosen as target groups for further investigation on the availability and reliability of the methods for their analysis.

2. List of regulated food additives and corresponding food categories with MLs different than *quantum satis*

The list was prepared from Commission Regulation (EU) No 1129/2011, amending Regulation (EC) No 1333/2008. In total 1355 food additive/matrix combinations were listed with 570 entries belonging to sweeteners and food colours. The lowest MLs (<10 mg/kg), which could be challenging for analytical determination, concern mainly the groups of sweeteners and colourants.

3. Present state-of-art of the methods for determination of sweeteners and colourants in food matrices with maximum level according to Commission Regulation (EU) No 1129/2011

3.1. Sweeteners

Regulation (EU) No 1133/2008, amended by Commission Regulations (EU) No 1129/2011 lists 16 sweeteners. Among them are 9 artificial sweeteners.

E-number	Name	E-number	Name
E 950	Acesulfame K	E 957	Thaumatin
E 951	Aspartame	E 959	Neohesperidine dihidrochalcone
E 952	Cyclamates	E 961	Neotame
E 954	Saccharins	E 962	Salt of aspartame-acesulfame
E 955	Sucralose		

Commission Regulation (EU) No 1129/2011 is amended by Commission Regulation (EU) N0 1131/2011, adding one natural intensive sweetener (E960-steviol glycosides) to that list.

Maximum levels were set for a number of food categories for ten of the 17 authorised sweeteners.

3.1.1. Official (standard) method for determination of sweeteners in foodstuffs

ISO, CEN, AOAC, Codex Alimentarius and Member States National Standardisation Institutes' databases were searched for standardised analysis methods for the determination of sweeteners in foodstuffs. The keywords used as search criteria were "method", "determination", "sweetener", "foodstuff", as well as all individual sweeteners names specified in 3.1.The outcome was collected in a table provided to EFSA.

Five EN standards cover eight of the sweeteners in a large variety of food matrices. The respective methods were validated by interlaboratory studies. National standard organisations in the EU countries plus Croatia, Iceland, Norway and Switzerland are bound to apply these European standards, aiming at ensuring the proper implementation of current legislation. All 37 European Standard Institutions adopted the EN Standards for sweeteners as their national standards. No additional national standard methods were found for the determination of any other approved sweetener.

No standard method exists for the determination of **thaumatin** (E 957) and the recently approved **steviol glycosides (E 960)** in any food commodities.

The only available standard method for **neotame** (E 961) (EN 15911:2010), based on HPLC/ELSD, is validated for beverages, yogurts and canned fruits starting from 38.4 mg/l (limit of quantification LOQ 28 mg/l). It is not applicable for lots of beverages and canned fruits with MLs in the range of 1-20 mg/kg, which is caused by the low sensitivity of the ELSD detector. Applicability should be verified for broader range of food categories.

The three European Technical Specifications (CEN/TS 14537:2003, CEN/TS 15606:2009) for the determination of **neohesperidine dihydrochalcone** (E 959) (NHDC), are validated for levels above 30 mg/l (kg) only. There is a need to evaluate the applicability of the methods for food categories with lower MLs – e.g. 5-20 mg/l(kg) for fats, meat, beverages and jams and marmalades.

The two standard methods for the determination of **cyclamates** (E 952i-iii) (EN 12857:1999 and EN 15911:2010) are applicable to different content ranges starting from 178.8 mg/kg and 28.3 mg/kg respectively. This is the consequence of the low UV absorption of cyclamates. Evaporative light scattering detection is applied for the lower content range. However, sensitivity issues should not provide any problem for the implementation of legislation, as the lowest MLs are set at 250 mg/kg.

For **acesulfame** (E 950), **aspartame** (E 951) and the **salt of aspartame-acesulfame** (E 962) the lowest ML is 25 mg/l (for beer and malt beverages). This is outside the validated concentration range of the three EN Standards. The applicability of the three EN methods EN 12856:1999; EN 15606:2009 (both based on HPLC/UV detection) and EN 15911:2010 (based on HPLC/ELSD and validated starting from 38.4 mg/l) needs therefore to be evaluated.

The lack of any chromophore in **sucralose** (E 955) makes a sensitive and specific detection by direct UV absorption difficult. Both recent EN 15911:2010 and EN 16155:2012 covering the determination of sucralose by HPLC/ELSD or HPLC/RI detection in a wide variety of food categories, are applicable for concentrations above 30 and 87 mg/kg(l) respectively. However, lower MLs were specified only for beer and malt beverages (10 mg/l).

For **saccharin** (E 954) the lowest ML is 80 mg/l (kg), which is covered by all three EN standards.

3.1.2. Other methods from the literature

Sweeteners may be used separately or in combination with other sweeteners, as so called blends. Nowadays, the common trend in food industry is to use sweetener blends, because some of the sweeteners cause side tastes and aftertastes that can limit their application in foods and beverages.

Consequently the method of choice for the determination of artificial sweeteners in different food matrices is HPLC because of its multi-analyte capability, compatibility with the physico-chemical properties of sweeteners, high sensitivity and robustness.

CE and IC are both interesting alternatives to HPLC. The resolving power of these techniques is in many cases comparable with that of HPLC and, frequently, their running costs are lower. However, it seems that due to limited robustness, in the case of CE methods, and the modest choice of separation mechanisms, in the case of IC, these methods are less popular. (Agata Zygler et al., 2009)

TLC and GC have been applied occasionally to the analysis of artificial sweeteners. TLC methods are characterized by poor separation efficiency and GC methods require derivatization that is time consuming and labour intensive

Robust and reliable analytical methods are essential to meet the needs of growing markets in quality control and consumer safety

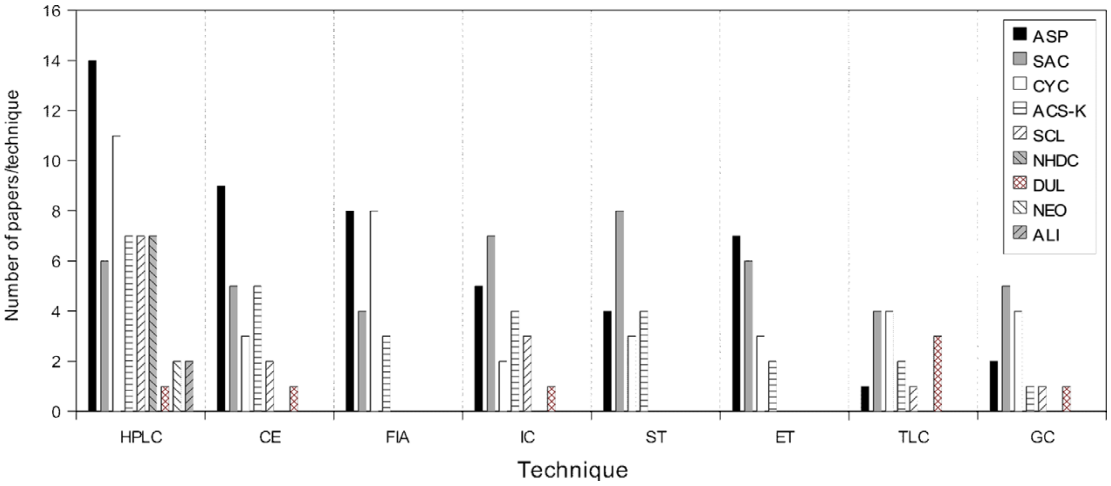

Figure 3: The use of analytical techniques to analyze artificial sweeteners. CE, Capillary electrophoresis; ET, Electroanalytical techniques; FIA, Flow-injection analysis; GC, Gas chromatography; HPLC, High-performance liquid chromatography; IC, Ion chromatography; TLC, Thin-layer chromatography; ST, Spectroscopic techniques (Agata Zygler et al., 2009).

As can be seen in Figure 3, commonly used sweeteners, with decades of history of usage (i.e. aspartame, saccharin, cyclamate and acesulfame-K), can be determined by all current analytical techniques. Among other recently introduced or approved sweeteners, sucralose receives the most attention, while a much smaller number of reports deal with the determination of neohesperidine dihydrochalcone, alitame and neotame. At present literature lacks reports with comprehensive information on method performance characteristics for the determination of thaumatin and steviol glucosides in any food category.

Since there are tens of possible sweetener combinations, performance of the methods, capable of determining several sweeteners in one run are to be evaluated. So far, the majority of the published multi-sweetener methods focused on the determination of just a few (3–4) compounds. Recently, however, some reports were published on methods covering a much wider range of artificial sweeteners (M.1.2.4, M.1.2.15-1, M.1.2.20).

With regard to food commodity, sweeteners are most frequently analysed in beverages, mainly soft drinks.

Sample preparation depends in general on the type of food matrix. Some samples can be analyzed directly or after minimal pre-treatment (dilution, filtration). However, extraction, clean up and/or purification might be necessary, depending on the complexity of the sample, in order to eliminate interferences.

SPE-based sample-preparation protocols seem to be the best available choice. They are simple, reproducible, reasonably quick and cost efficient. They are universal and compatible with the most common measurement techniques used in food analysis. Other sample preparation procedures are possible, but usually they are highly specific to the analyte under study, the sample or the technique of final determination

The studied papers indicate since recently a trend towards the use of LC/MS techniques for the simultaneous determination of several sweeteners and even some other food additives as preservatives and colours.

Zygler et al. (2011) in-house validated a method based on solvent extraction, SPE cleanup, LC/ESI-MS for determination of nine EU authorised sweeteners for a broad range of food matrices such as beverages (beers, carbonated, non-carbonated, juices), dairy (yoghurt) and fish (marinades, salads, pastes) with LOQ of 0.5-2.5 mg/l(kg) and working range starting from 5 mg/l.

No method could be found in literature for the determination of **thaumatin** (E 957) and the recently approved **steviol glycosides** (E 960) in any food commodities. Their chemical nature also would make inclusion in multianalyte methods difficult.

Thaumatin, an intensely sweet basic protein, is primarily used for its flavour modifying properties and not exclusively as a sweetener. According to some authors (Wasik et al., 2007) methods used for the determination of thaumatin are based on immunochemical assays. Since thaumatin is approved for use only as flavour enhancer, its MLs are generally low. It may be applied in a limited number of food categories (only 15 food sub-categories) and has the lowest ML of all regulated sweeteners (in non-alcoholic flavoured beverages). However, as there is not any well characterised method available to determine thaumatin at relevant levels in food, further development is needed in order to fill this gap.

Steviol glycosides are since recently authorised commercial sweeteners. The products consist of up to ten different steviol glycosides, with stevioside and rebaudioside A as major components. The newly revised specifications cover a range of compositions that include products of not less than 95 % stevioside, rebaudiosides A, B, C, D, E and F, steviolbioside, rubusoside and dulcoside on the dried basis.

All of the applicants for autorisation of steviol glycosides indicated that in-house validated HPLC methods were available for the identification of stevioside, rebaudioside A and other related steviol glycosides in food and beverages, including beverages with and without alcohol. HPLC methods have been recently published (Geuns et al., 2008a; Gardana et al., 2010) for the determination of steviol-glycosides or steviol in Stevia leaves and a commercial table top sweetener (Truvia®), which may serve as a base for further method development for determination of steviol glycosides in different food categories.

Concerning other sweeteners, gaps were identified concerning the determination of neohesperidine dihydrochalcone and neotame in some regulated food matrices, particularly salts, spices, soups, sauces, salads and protein products. For neohesperidine dihydrochalcone an additional gap was identified for the group snacks and processed nuts.

Sweeteners monitored by MS are acesulfame K, aspartame, cyclamic acid or cyclamates (Na and Ca salts), saccharin and its Na, K and Ca salts, and neohesperidin dihydrochalcone. Data is not available for thaumatine, neotame, sucralose and steviol glucosides, indicating that these sweeteners are not included in the control program of the MS. Hence there is at least a gap in the occurrence database. The lack of data might indirectly indicate potential problems in their analytical determination.

3.2. Food colours

The list of food colours authorised for use in EU according to the Regulations (EU) No 1333/2008, amended by Commission Regulation (EU) No 1129/2011 can be separated into two main groups of substances which differ substantially in their nature, structure, and chemical characteristics – natural colours and artificial colours (synthetic food colours).

3.2.1. Synthetic food colours

The group of artificial food colours (synthetic food colours) consists of:

E-number	Name	E-number	Name
E 102	Tartrazine	E 129	Allura Red AC
E 104	Quinoline Yellow	E 131	Patent Blue V
E 110	Sunset Yellow FCF/Orange Yellow S	E 132	Indigotine, Indigo carmine
E 122	Azorubine, Carmoisine	E 133	Brilliant Blue FCF
E 123	Amaranth	E 151	Brilliant Black BN, Black PN
E 124	Ponceau 4R, Cochineal Red A	E 155	Brown HT
E 127	Erythrosine		

Synthetic food colours guarantee intensive and permanent colour of food products. The costs of production are much lower in comparison with natural food colours. These advantages favour their application from the technical point of view.

Many analytical methods were developed for the determination of synthetic food colours in food, such as: thin-layer chromatography; spectrometry, voltammetry; differential pulse polarography; capillary electrophoresis; high performance liquid chromatography (HPLC) and ion chromatography. Kucharska and Grabka (2010) reviewed recently chromatographic methods for the determination of synthetic food colours.

The easiest chromatographic technique for the analysis of dye mixtures is thin layer chromatography (TLC). It is the best solution for qualitative analysis, providing satisfactory results in short time. ISO standardised a TLC based method for the detection of synthetic food colours in meat and meat products:

ISO 13496:2000 Meat and meat products - Detection of colouring agents - Method using thin-layer chromatography

A wide variety of methods was published for the quantitative analysis of synthetic food colours. They are based on CE (M.2.2.13, M.2.2.28, M.2.2.30, M.2.2.31) and HPLC (M.2.2.01, M.2.2.02, M.2.2.03, M.2.2.04, M.2.2.05, M.2.2.10, M.2.2.11, M.2.2.12), covering a large range of foodstuffs. In recent years a tendency is observed to use more sophisticated analytical techniques such as HPLC hyphenated to mass spectrometry (M.2.2.06, M.2.2.08, M.2.2.09, M.2.2.29).

The methods listed in the database were subjected to different levels of single laboratory validation, which is reflected in the classification as "Methods approved for analysis" and "Methods assumed suitable for analysis". Methods performance data is not complete for most of the reported methods -

e.g. instrument detection limits provided instead of LODs, recovery checked only with some matrices, not covering the whole scope, or tests at only one spiking level.

Single laboratory method validation data was satisfactory in three cases only (M.2.2.20, M.2.2.30 and M.2.2.34). All other methods would require additional characterisation to fill the gaps. However, the data provided allow the assumption of applicability of the method for the particular scope, e.g. LOQs were lacking in the paper, but reported instrumental detection limits were far below the lowest MLs. Hence it was assumed that the respective method might be able to achieve LOQs that are below the MLs.

As the ionisation efficiencies of some water-soluble colourants are very low, the LODs of some colourants obtained by tandem mass spectrometry (MS/MS) were reported up to 10 times higher then for diode array detection (DAD) (M.2.2.9), but even in that case the LOQs were sufficiently below the corresponding MLs.

There are 121 synthetic colorant /matrix combinations with ML between 10-500 mg/l(kg) for which methods were searched in the literature. Methods "approved" or "applicable" are compiled to the database for all synthetic colourants and food categories except for the regulated E 131 Patent Blue and GreenS (E 142).

Serious problems for regulatory compliance assessment are provided by the group of colours with combined maximum limits. Fourteen synthetic colourants and 2 natural colours are included in that group and combined MLs for 38 food sub-categories ranging from dairy, confectionery, meat, fish, cereals, spices, soup, sauces, to beverages, with MLs between 50 and 500 mg/kg are laid down in legislation. Hence, each sample has to be subjected to a number of different analysis methods for monitoring of the content of substances that are covered by the combined MLs. The method performance database contains data on methods which cover at least most of the substances for which combined MLs were established (Group III substances). However, methods are not available for all of the more than 600 food colour/food subcategory combinations. Methods are missing for E 155 Brown HT (E 155), which makes part of Group III. So, the combined MLs of Group III substances cannot be controlled properly by chemical analysis, and the reliability of occurrence data for this group of substances would be questionable.

3.2.2. Natural food colours

Natural food colours could be grouped into nine main groups, which related to the main chemical classes:

(1) Curcumin (E 100).

(2) Riboflavins (E 101i–ii).

(3) Cochineal – including carminic acid (E 120).

(4) Chlorophylls – including chlorophyllins and copper analogues (E 140–141).

(5) Caramels – Classes I–IV (E 150a–d).

(6) Carotenoids – (E 160a–f, E 161b, E 161g).

(7) Beetroot red, betanin (E 162).

(8) Anthocyanins (E 163).

(9) Others: Vegetable carbon (E 153), Calcium carbonate (E 170), Titanium dioxide (E 171) and Iron oxides and hydroxides (E 172).

Analysis procedures were established for the analysis of synthetic food colourants. The issue is more complex for **natural food colours,** which are partially weakly defined from the chemical point of view (e.g. E 150a-d caramels).

The diversity of the chemical classes of natural food colours, the complexities of their structures and the wide range of applications in foods and beverages present analytical challenges that in turn requires a diverse range of analytical procedures.

(1) Curcumin (E 100).

Methods for the determination of curcumin in foods are scarce. A number of sensitive analytical methods have been developed for the determination of curcumin in various biomatrices and in turmeric root in order to study their biological effects and pharmacological properties (Scotter, 2009).

Most modern methods (M.2.2.14, M.2.2.15, M.2.2.16, M.2.2.35) for the determination of curcumin in food are able to achieve limits of quantitation below 20 mg/kg, by using HPLC with photodiode array and fluorescence detection. Extraction conditions are generally very simple and various clean-up techniques have been reported, but have not been validated for all regulated foodstuffs.

Methods must be capable of detecting and quantifying all three main curcuminoids; curcumin (CUR) along with its demethoxy (DMC) and bisdemethoxy (BDMC) analogues. Therefore, the access to suitable reference materials for the main colouring principles is crucial (Scotter, 2009).

(2) Riboflavins (E 101i–ii).

Annex II of Regulation (EU) 1129/2011 specifies for riboflavins maximum limits of 100 mg/kg in alcoholic beverages and *quantum satis* in a number of foodstuffs such as meat, fish and preserved red fruits. High performance thin layer chromatography hyphenated to mass spectrometry (M.2.2.25) was suggested for the analysis of riboflavins in beverages. Despite satisfactory performance characteristics, the applicability in routine control laboratories is limited due to the required instrumentation.

A less specific analytical technique for determining the riboflavin content of foods comprises acidic followed by enzymatic hydrolysis of the sample and measurement by reverse-phase HPLC with fluorimetric detection (Arella et al., 1996; Tang et al., 2006). While the performance of the HPLC part is well established, providing adequate sensitivity and selectivity, the hydrolysis steps might introduce bias. They were reported to be time-consuming and varying in efficacy depending upon the enzymes used and the conditions employed (Ndaw et al., 2000). Extraction and hydrolysis conditions require additionally adaptation to the respective sample type.

Published methods, such as the one described by van den Berg et al. (1996), are adequately sensitive for monitoring of the levels of riboflavin added to food for colouring purposes. They could form the basis for the development of a method that includes in its scope all foods, for which the application of riboflavin was approved.

(3) Cochineal – including carminic acid (E 120).

Annex II of Commission Regulation (EU) No 1129/2011 lists for E 120 maximum limits of between 100 and 250 mg/kg for number of foodstuffs as diary products, meat, fish, beverages, fruits and

vegetbles. Additionally it may be used in a number of food categories *ad quantum satis*. Most modern published methods are able to achieve for E 120 limits of quantitation below 1mg/kg using HPLC with DAD, UV-VIS or fluorescence detection (Scotter, 2009). A number of methods for the determination of E120 in foodstuffs were reasonably well established and validated for different sample types. Reversed phase HPLC with UV-VIS detection is the preferred measurement technique (M.2.2.11, M.2.2.12, M.2.2.13, M.2.2.26, M.2.2.32, M.2.2.34), but CE (M.2.2.31) EIA (M.2.2.33) and LC-MS/MS (M.2.2.29) methods were proposed as well.

(4) Chlorophylls – including chlorophyllins and copper analogues (E 140–141).

Chlorophylls are permitted *ad quantum satis* in a number of foodstuffs

The chemistry of chlorophyll and its many analogues is complicated and requires the extraction, separation, identification and quantitation of several components. Suitable specific marker compounds need to be identified in order to make the methods more readily transferable to enforcement laboratories (Scotter, 2011). This in turn requires the preparation of suitable reference materials.

Analytical methods might need further development to broaden their scope to both native and coppered analogues, as well as to all food categories specified in the regulation. Conditions for the extraction and clean-up are reasonably simple but are likely to require refinement to accommodate the increased scope. Methods based on HPLC-PDA and HPLC-FLU (Scotter et al., 2005) and LC-MS/MS (Mortensen and Geppel, 2007) could be considered as a basis for any future development and validation, since they offer adequate selectivity and sensitivity for the detection and quantitation of the main chlorophyll/copper chlorophyll analogues. It is also assumed that regulatory compliance is not an issue since chlorophylls are permitted *ad quantum satis* (Scotter, 2011).

(5) Caramel colours – Classes I–IV (E 150a–d).

Caramel colours are permitted in a number of foodstuffs *ad quantum satis*. Caramel colour preparations represent a complex mixture of compounds that can be approximately divided into high and low molecular weight fractions (HMW and LMW respectively). HMW caramel fractions can be characterised using electrochemical methods such as electrophoresis, but also by methods based upon the differentiation of the complex molecules according to their molecular weight/shape, such as gel permeation chromatography and ultrafiltration. HPLC and GC have been used to characterise the LMW fraction but relatively few compounds have been identified. Most analytical studies on caramel colours have therefore centred on method-driven characterisation, i.e. ‘fingerprinting’ of subfractions (Scotter, 2011).

Burch et al. (2007) have recently reviewed the extraction of caramel colours from complex food matrices. It is clear that the identification of specific marker compounds for both the qualitative and quantitative analysis of caramel colours in foodstuffs is a prerequisite for any monitoring activity. The methods described by Coffey et al. (1997), Royle and Radcliffe (1999), Ames et al. (2000), Aulenta et al. (2001), Wood et al. (2002) and Burch (2005) could be used as a basis for any future development of extraction protocols. Both HPLC and CZE analysis may be considered for separation and measurement of caramels. Anyhow regulatory compliance is assumed for approved food categories, as caramels are permitted *ad quantum satis*.

(6) Carotenoids – (E 160a– f, E161b, E 161g). The group of carotenoids consists of:

E 160a	Carotenes
E 160b	Annatto
E 160c	Paprika extract, capsanthin, capsorubin
E 160d	Lycopene
E 160e	Beta-apo-8′-carotenal (C 30)
E 161b	Lutein
E 161g	Canthaxanthin (*)

(*) authorised only for medicinal products

Significant effort has been made by the food industry to use carotenoids as colourants in foods, not only as a replacement for artificial colours but also to add value to the products due to the health benefits associated with carotenoids (Scotter, 2011). Consequently, the vast majority of available methods for the extraction and analysis of carotenes are focused on the determination of specific carotenoids from fruit, vegetable and algal sources. There is relatively little information on methods for their determination in foodstuffs in which they do not occur naturally.

Only few methods were found on the determination of carotenoids in processed foods and beverages, and even fewer on the analysis of carotenoids acting as food colours (M.2.2.23, M.2.2.24, M.2.2.36). The analytical methods published so far (M.2.2.22, Dias et al., 2008 and Serino et al., 2009) will require adaptation of their scope to fully cover carotenoids used as colourants.

One of the main issues hampering the analysis of carotenoids is the availability and stability of reference materials. Their inherent instability requires storage under inert conditions and regular checks of the integrity of the reference materials. However, according to Scotter (2011) most of the main carotenoids are available commercially but at high costs only.

Annatto (E 160b)

Published methods for annatto are adequately sensitive for the levels of annatto added for colouring purposes. HPLC methods must be capable of detecting and quantifying all of the main bixin and norbixin isomers. However, as for other natural colorants, the access to well characterised reference materials for the main colouring components is crucial (Scotter, 2011).

Methods for the determination of annatto in a variety of foodstuffs are reasonably well established and have been validated for a number of different sample types (M.2.2.19, M.2.2.21). Conditions for the extraction and cleanup are sample dependent and require refinement to widen the scope of the methods to all foodstuff covered by EU regulations. The methods reported by Scotter et al. (2002) M.2.2.17, Breithaupt (2004) M.2.2.22, and Noppe et al. (2009) M.2.2.21 could be considered as a basis for future method development and validation.

(7) Beetroot red, betanin (E 162).

Very few literature references were found on the extraction of added beetroot red colour from foodstuffs.

Methods published so far do not provide sufficient data to assess their suitability for the detection of E 162 in foodstuffs at relevant levels. However, according to Scotter (2011) several RP-HPLC methods with UV-VIS (PDA) and MS detection might be adaptable for food monitoring purposes. Analytical procedures described by Kujala et al. (2001) and Stintzing et al. (2006) are good candidates for further development. Procedures for the extraction, purification and characterisation of reference materials might also require development. However it could be presumed (Scotter, 2011) that regulatory compliance in terms of added levels is not an issue since E162 is permitted *ad quantum satis* in most food categories with the only exception provided by breakfast cereal.

(8) Anthocyanins (E 163).

Since the vast majority of available methods for the extraction and analysis of anthocyanins are focused on the isolation, purification and identification of specific analogues from a wide range of food sources, there is very little information available on methods for their determination in foodstuffs containing added anthocyanins. It is not clear therefore to what extent naturally occurring anthocyanins will interfere in the determination of added anthocyanins. However, this might be relevant only for fruit flavoured breakfast cereals, as anthocyanins are permitted *ad quantum satis* in the other regulated food categories.

The methods described by Goiffon et al. (1999), Tian et al. (2005), Lee et al. (2008) and Ling et al. (2009) could be considered for use as a basis for any future development if needed.

(9) Others – Vegetable carbon (E 153), Calcium carbonate (E 170), Titanium dioxide (E 171) and Iron oxides and hydroxides (E 172). The study does not target the inorganic food colours as they are permited in different food categories a*d quantum statis.*

In summary, there are 100 natural colour/food category combinations with MLs different from *quantum satis*. 74 of them concern three natural colours: E 120 cochineal, carmines (19); E 160b annatto (21) and E 160d lycopene (34). Analytical methods were proposed for all three of them, which might serve as basis for further development, improvement and validation. This will especially be necessary for the determination of annatto in beverages and lycopene in meat and fish. Other gaps in the availability of analysis methods concern E 161b (lutein) in fish as well as E 162 (beetroot red, betanin) and E 163 (anthocyanins) in breakfast cereals.

4. Technical specifications

Two documents were drafted covering the technical details to be included in possible future calls for ad-hoc projects to address the identified gaps in current MS post-market monitoring activities. One technical specification relates to projects on ad-hoc surveys on food additives whereas the other contains technical details for projects on analytical method development/improvement. The two documents are based on EFSA templates. Information that needs to be provided by either EFSA or the applicant of future projects is indicated in the templates. They are designed to allow EFSA specifying potential projects from very general to very specific.

The technical specifications cover for both types of research projects the objectives, milestones and deliverables, staff requirements, and award criteria. A timetable specifies for the different milestones of the projects the period available for the development of the respective deliverables.

The applicants for projects will be required to provide in their tender the scientific basis for any experimental design either specified in the tender, or foreseen to be developed during the execution of

the project. The authors of this report believe that EFSA shall have at any time control over the execution of the projects. Therefore, it was specified that the contractor has to seek the agreement of EFSA prior to commencing crucial steps of the project.

Other key points were that developed analytical methods must be broadly applicable in food control laboratories, which shall exclude the proposal of exclusive instrumentation in the design of the analytical method. The proposed instrumentation must be available to the contractor at the start of the project. A high level of experience of involved staff in the execution of the analytical method(s) and operation of the analytical instrumentation used in the project is also required prior to the start of the project, as the duration of the project(s) is usually too short for training staff during the execution phase.

Special emphasis was given to the broad acceptance of the protocols/procedures applied in the project for e.g. method validation.

The specifications of strict quality control measures shall support the reliability of the developed methods/gained monitoring data, and consequently any conclusion EFSA would draw on basis of the provided data. EFSA might even wish to foresee external review of the gained data, especially if the project would concern a politically sensitive topic.

The two draft technical specifications were provided to EFSA together with this report.

CONCLUSIONS

- The groups of sweeteners and food colours were identified as priority substances to be addressed in post market monitoring. Filling gaps in analytical methodology should be directed to these groups of food additives.
- Internationally accepted standard methods do not exist for the determination of **thaumatin** (E 957) and the recently approved **steviol glycosides** (E 960) in any food commodities. Methods for the determination of thaumatin are also lacking in the scientific literature.
- The applicability of the European Standard for the determination of **neotame** (E 961) (EN 15911:2010) should be verified for a broader range of food categories and should especially focus on those for which maximum permitted levels (ML) at levels below the limit of quantification (LOQ) of the method are specified.
- Gaps in the analytical methods (method performance) database were also identified for **neohesperidine dihydrochalcone** (E 959) and **neotame** (E 961)**,** which do not yet cover some of the regulated food matrices.
- No standard method is available for the quantitative determination of approved **food colours (natural or synthetic)** in any food commodity.
- Methods were found in literature for all synthetic colours and food categories except for **Patent Blue** (**E 131)** and **GreenS (E 142)**
- Serious problems for monitoring/regulatory compliance assessment could be provided by the group of **food colours with combined maximum limits** as analytical methods do not yet cover all food colours in that group. In addition the development of multi-analyte analysis methods allowing the determination of all food colours with combined maximum limits in a single run would be favourable for occurrence assessment in a broad range of colour/food commodities combinations.
- Four groups from the natural food colours - **Chlorophylls – (E 140–141); Caramels – Classes I–IV (E 150a–d); Beetroot red, betanin (E 162) and Anthocyanins (E 163)**- are permitted for use *ad quantum satis* in most food categories. Hence compliance assessment is not required for most food groups. However, it should be evaluated whether there is a need for development of methods for the determination of beetroot red (E 162) and anthocyanins (E 163) in breakfast cereals, for which MLs were specified.
- Although available in the literature, there is room for further improvement in the analytical methods for the following groups of natural food colours - Curcumin (E 100); Riboflavins (E 101i–ii), Cochineal – including carminic acid (E 120), Carotenoids – (E 160a–f, E 161b, E 161g). The vast majority of available methods for the extraction and analysis of natural food colours are focused on the isolation, purification and identification of specific analogues from a wide range of food sources. There is very little information on methods for their determination in foodstuffs containing added natural food colours. Key problems concern availability of reference standards with known purity for the main colouring principle and isolation of the food colours from different complex foodstuffs.

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APPENDIX I - LAYOUTS OF TABLES FOR COLLECTING METHOD PERFORMANCE DATA

gories and methods to control ML

Category number					E-number	Name	Maximum level (mg/l or mg/kg as appropriate)	Restrictions/exceptions	Methods approved for analysis
oducts and es	01.4	Flavoured fermented milk products including heat-treated products			E 950	Acesulfame K	350	only energy-reduced products or with no added sugar	M.1.1.2; M.1.1.5; M.1.2.15; M.1.2.18
ices					E 950	Acesulfame K	800	only energy-reduced or with no added sugar	M.1.2.18
d vegetables	04.2	Processed fruit and vegetables	04.2.2	Fruit and vegetables in vinegar, oil, or brine	E 950	Acesulfame K	200	only sweet-sour preserves of fruit and vegetables	M.1.2.9, M.1.2.15-1

rences to standards/publications;

Autors	Article name		Journal
Kang Maa, Ya Nan Yang, Xiao Xiong Jiang, Min Zhao, Ye Qiang Cai	Simultaneous determination of 20 food additives by high performance liquid chromatography with photo-diode array detector	2012	Chinese Chemical Letters, 23, Issue 4, April 2012, Pag 495

pe of the method and performance indicators

Additives	matrix	Method	Range	repeatability	reprodicibility	Horrat value	LOD	LOQ
				r, %	R, %		mg/l(kg)	mg/l(kg)
acesulfame-K	canned fruits (pears)	SPE, HPLC/ELSD	38,4 - 391,3 mg/kg	2,9 - 6,9 (4 level)	4,5 - 14,8 (4 level)	0,7 - 1,6	13*	29*

ABBREVIATIONS

EFSA	European Food Safety Authority
JRC	Joint Research Centre
AOAC International	Association of Analytical Communities
CEN	European Committee for Standardization
ISO	International Organization for Standardization
RASFF	Rapid Alert System for Food and Feed
LOD	Limit of determination
LOQ	Limit of quantification
ML	Maximum permitted level
CE	Capillary electrophoresis
DAD	Diode array detector
ELSD	Electro-light scattering detector
GC	Gas chromatography
HPLC	High performance chromatography
IC	Ion chromatography
LC-MS/MS	Liquid chromatography with tandem mass-spectrometry
MS	Mass spectrometry
PDA	Photodiode array detector
RI	Refractive index detector
TLC	Thin layer chromatography
UV-VIS	Ultraviolet-visible spectrometry