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PhD Thesis

MASS SPECTROMETRY APPROACHES FOR EVALUATION OF DIETARY EXPOSURE TO TOXIC CONTAMINANTS

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ABSTRACT

The legislation on environmental contaminants in food and feed chain is constantly evolving. Some of these contaminants, such as mycotoxins, have already been the subject of official control of the food and feed chain, while others, such as cyanotoxins compounds, are not yet covered.
Present work is based on the development of innovative techniques aimed at the implementation of confirmatory analytical methods for the control of substances present and of probable future inclusion in the Official monitoring plans, i.e. aflatoxin M_1 in dairy products, T-2 and HT-2 toxins in feedingstuffs and microcystins and cylindrospermopsin in particular products such as dietary supplements.
Regarding the mycotoxins, this work aims to focus on validation study of an analytical confirmatory method for the detection of T-2 and HT-2 toxins in feedingstuffs, as required by Commission Recommendation n 165 of 27 March 2013 with more rapid and reliable extraction methods based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) extraction kit coupled to detection in LC-MS/MS.
The applicability of QuEChERS extraction method was also tested for the analysis of AFM_1 in dairy products with final determination by LC-ESI-Ion Trap MS/MS.
As to cyanotoxins, the Committee of Experts of the WHO defined a tolerable daily intake only for one of the up to 90 known microcystins, i.e. microcystin LR, although recent studies indicate that other congeners are eventually more toxic; here we present the development of a fully validated method for a rapid and reliable detection of these compounds in dietary supplements with ESI-Ion Trap MS/MS detection.
So this work has allowed us to achieve several goals. Firstly, it has been provided an example, so far not present in the scientific literature, of application of the QuEChERS extraction analysis for a fully validated confirmatory method for T-2 and HT-2 determination in feedingstuffs, whose validity has been proved fullfilling European Legislation criteria requirements; moreover, for the confirmation of the presence of aflatoxin M_1 in dairy products in compliance with the needs of a quick response, an analytical solution to laboratories appointed for Official Control was provided.
Moreover, the development and validation of analytical methods for substances such as microcystins characterized by toxicity and evident presence in dietary supplements, which are not covered by national and European legislation allowed us to provide guidance to the regulation both for performance criteria and for safety levels. The presence in dietary supplements of another cyanotoxin, the cylindrospermopsin was also evidenced.

Keywords: mycotoxins, cyanotoxins, mass spectrometry

RIASSUNTO

La legislazione riguardante i contaminanti negli alimenti destinati all'uomo ed agli animali è in costante evoluzione. Alcuni di questi contaminanti, come le micotossine, sono già da tempo soggetti al controllo nella catena degli alimenti e dei mangimi, mentre altri, come le cianotossine non sono ancora contemplati.
Questo lavoro si basa sullo sviluppo di tecniche innovative con lo scopo di implementare i metodi di conferma analitici per il controllo di sostanze presenti nei piani del controllo ufficiale o di probabile prossima inclusione, cioè l'aflatossina M_1 nei prodotti caseari, le tossine T-2 e HT-2 nei mangimi e le microcistine e la cilindrospermopsina in prodotti particolari quali gli integratori alimentari.
Riguardo le micotossine, questo alvoro ha lo scopo di focalizzarsi su uno studio di validazione di un metodo di conferma analitico per le tossine T-2 e HT-2 nei mangimi, come richiesto dalla Raccomandazione della Commissione n 165 del 27 Marzo 2013 con un metodo di estrazione più rapido e affidabile basato sul kit di estrazione QuEChERS (Quick (rapido), Easy (facile), Cheap (economico), Effective (efficiente), Rugged (robusto), e Safe (sicuro)) accoppiato da determinazione in LC-MS/MS.
L'applicabilità del metodo di estrazione QuEChERS è stata anche testata per l'analisi della AFM_1 in prodotti caseari con determinazione finale in LC-ESI-Ion Trap MS/MS.
Per quanto rigurada le cianotossine, la Commissione di Esperti dell'OMS ha definito la dose quotidiana tollerabile solo per una delle oltre 90 microcistine note, cioè la microcistina LR, benché studi recenti indichino che altri congeneri sono eventualmente più tossici; in questa sede viene presentato lo sviluppo di un metodo completamente validato per una determinazione rapida e affidabile di questi composti negli integratori alimentri con determinazione in LC-ESI-Ion Trap MS/MS.
Questo lavoro ha consentito di raggiungere diversi obbiettivi. Primo, è stato fornito un esempio, finora non presente nella letteratura scientifica, dell'applicazione dell'analisi di estrazione QuEChERS per un metodo di conferma completamente validato per le tossine T-2 e HT-2 nei mangimi, che è stato provato soddisfare i criteri di prestazione richiesti dalla legislazione europea; inoltre, per la conferma della presenza di aflatossina M_1 nei prodotti caseari in accordo con le esigenze di una risposta rapida, è stata fornita una soluzione analitica ai laboratori preposti al controllo ufficiale.
Inoltre, lo sviluppo e la validazione di metodi analitici per sostanze come le microcistine, caratterizzate da tossicità e dall'evidente presenza negli integratori alimentari, che non sono contemplate dalla legislazione europea e nazionale, ci ha consentito di fornire indicazioni per la regolamentazione sia per i criteri di prestazione che per i limiti di sicurezza. È stata evidenziata la presenza negli integratori alimentari di un'altra cianotossina, la cilindrospermopsina.

Parole chiave: micotossine, cianotossine, spettrometria di massa

PREFACE

0. Secondary methabolism derived toxins

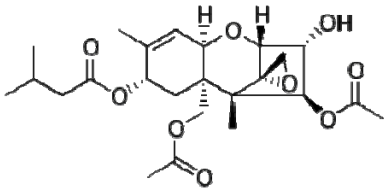
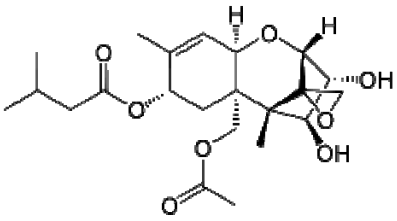
Secondary metabolites are generally defined as compounds that are not essential for growth or survival of the producing organism and are generally produced following active growth, however this is not true in all cases (pigments, sideophores and pheromones). The production of these metabolites is tightly regulated and dependent on the immediate environment and developmental stage of the producing organism. The group includes both simple molecules such as alcohols, sugars and organic acids; and complex compounds such as polyketides, flavonoids, terpenes and non-ribosomal peptide compounds (Medentsev & Akimenko 1998) and many have an unusual chemical structure.
The occurrence of large scale secondary metabolites is not common to all living organisms but restricted to certain taxonomic groups.
A characteristic feature of the most prolific producers of secondary metabolites among microorganisms is their adaptability to changing environmental conditions such as substrate, pH, temperature, nutrient, oxygen and water availability. The ability to use a wide variety of substrates of different compositions which enter primary metabolism at different points leads to an enlargement of the available pool of secondary metabolites.
Some secondary metabolites are toxic to humans and other animals. Yet others can modify the growth and metabolism of plants. Interestingly, the most important secondary metabolites seem to be synthesized from one or a combination of three biosynthetic pathways: polyketides arising from acetyl coenzyme A, mevalonate pathway that also arises from acetyl coenzyme A, and from amino acids. In addition, genes for the synthesis of some important secondary metabolites are found clustered together, and expression of the cluster appears to be induced by one or a few global regulators.
A rich and extensive secondary metabolism has for a long time been observed in bacteria and fungi. A variety of fungi produce a wide variety of secondary metabolites, some of which are potent toxins called mycotoxins.
Also cyanobacteria are prolific producers of natural secondary metabolites. Investigations into the biochemistry responsible for the formation of these compounds have revealed fascinating mechanisms that are not, or only rarely, found in other microorganisms.
Synthesized nonribosomally, the microcystins contain a number of unusual amino acid residues including the beta-amino polyketide moiety Adda (3-amino-9-methoxy-2,6, 8-trimethyl-10-phenyl-4,6-decadienoic acid), in addition, microcystins contain two amino acids that are linked with their ω-carboxy group in the peptide chain, namely glutamate and aspartate.
Produced by different genera of freshwater cyanobacteria, cylindrospermopsin is a polyketide-derived alkaloid.
Characteristic features of cylindrospermopsins include a guanidine moiety and a hydroxymethyluracil attached to the tricyclic carbon skeleton.
As these substances are toxic to human and animals, it is important to control their presence in the environment, in food and feed, so analytical methods are needed. In this work we want to develop analytical methods to detect different types of toxic secondary metabolites in different complex commodities with techniques based on mass spectrometry and use these to evaluate the presence of toxins in real samples.

0.1 Among mycotoxins: T-2 and HT-2 toxins

Mycotoxins are toxic secondary metabolites naturally produced by molds (fungi) that may contaminate agricultural commodities when environmental conditions are favourable. Because molds are present in soil and plant debris, and are spread by wind currents, insects, and rain, they are frequently found in/on foods together with their associated mycotoxins (Pittel, 2001).
Mycotoxins are produced by fungi or micromycetes according to four different pathways (Le Bars et al. 1996) involving secondary fungal metabolism, the bioconversion of plant compounds (dicoumarol), the reactivity of the plant to fungal aggression (coumestrol), and plant-fungus associations (for endophytic fungi) respectively.
Secondary metabolism differs from primary metabolism in the random nature of its activation, the diversity of the compounds formed, and the specificity of the strains involved. Secondary metabolism is not associated with cell growth, but instead generally responds to signals from the environment of the fungus. Whereas primary metabolism is common to all fungal species and interacts with the general metabolic pathways for the synthesis and catabolism of carbohydrates, lipids and proteins, secondary metabolism may be specific to a species, or even to a fungal strain and its genetic characteristics (Steyn et al. 1998).
The fungal contamination of plants and the bio-synthesis of toxins depends on environmental conditions: the state of the health of the plant before harvest, meteorological conditions, harvesting techniques, delays and hydrothermal conditions before stabilisation for conservation.
Six groups of mycotoxins are produced by the three principal genera of fungi *Aspergillus*, *Penicillium* and *Fusarium*. Mycotoxins display a diversity of chemical structures, accounting for their different biological effects. Depending on their precise nature, these toxins may be carcinogenic, mutagenic, teratogenic, oestrogenic, neurotoxic or immunotoxic. In animals, five mycotoxins — aflatoxins B_1 and aflatoxins M_1, ochratoxin A, moniliformin and sterigmatocystin—have been demonstrated to be carcinogenic. In humans, aflatoxins, and recently

ochratoxin A (1993), have been classified as carcinogenic by the International Agency for Research on Cancer. Fumonisin B1 is currently classified as a possible carcinogen and may soon be recognised as a proven carcinogen. Recent toxicological results for patulin have also suggested that this toxin may present a health hazard.

Table 1. T-2 and HT-2 toxins

Genera of fungi	Chemical structure of toxins
Fusarium graminearum, F. sporotrichioides, F. poae and F. equiseti	T-2 toxin $C_{24}H_{34}O_9$ 
	HT-2 toxin $C_{22}H_{32}O_8$ 

0.1.1 T-2 and HT-2 distribution

T-2 toxin (Table 1) is one of a group of trichothecene mycotoxins produced by various species of *Fusaria,* including *Fusarium sporotrichioides.* These fungi are routinely found on commodities such as wheat, maize, oats, barley, whereas it seems that rice is not interested by this kind of contamination. A related compound, HT-2 toxin (Table 1), is thought to be produced by the deacetylation of T-2 toxin by microflora (Desjardins et al. 2006).

0.1.2 T-2 and HT-2 toxicity

The toxicological effects of T-2 toxin and HT-2 toxin have been summarized in reports by the Joint Food and Agricultural Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives and the Council for Agricultural Science and Technology (FAO/WHO, 2001, Richard et al. 2002). T-2 toxin is a potent inhibitor of DNA, RNA and protein synthesis, and shows immunomodulatory and cytotoxic effects both *in vivo* and *in vitro* (Visconti et al. 1991). The toxicities of T-2 and HT-2 toxins are quite similar (FAO/WHO, 2001). *In vitro* human experiments have shown that T-2 toxin is rapidly metabolized to HT-2 toxin and, consequently, the toxicity of T-2 toxin *in vivo* might partly be attributed to HT-2 toxin (Königs et al. 2009).
The CONTAM Panel established a group tolerable daily intake (TDI) of 100 ng/kg b.w. for the sum of T-2 and HT-2 toxins. Estimates of chronic human dietary exposure to the sum of T-2 and HT-2 toxins based on the available occurrence data are below the TDI for populations of all age groups, and thus not an immediate health concern.

0.1.3 T-2 and HT-2 legislation

Since 2006, data on the presence of T-2 and HT-2 toxins in products intended for animal feeding were very limited and there were also an urgent need for the development and validation of a sensitive method of analysis. However there were indications that the presence of T-2 and HT-2 in products intended for animal feeding could be a reason for concern. Therefore, it was necessary to develop sensitive methods of analysis, collect more occurrence data, and carry out further investigations and research into the factors involved in the presence of T-2 and HT-2 in cereal and cereal products, in particular in oats and oat products.
In the Commission Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding are summarized the conclusions of the scientific opinions regarding the lack of reliable data on T-2 and HT-2 toxins, together with the large year-to-year variation in occurrence of these mycotoxins, in addition to the data already available from the coordinated control programmes for 2002 (Commission Recommendation 2002/214/EC of 12 March 2002), 2004 (Commission

Recommendation 2004/163/EC of 17 February 2004) and 2005 (Commission Recommendation 2005/187/EC of 2 March 2005).
One of the overall conclusion was that was appropriate to collect more data on these mycotoxins in the different feed materials and feedingstuffs and it was also appropriate to recommend guidance values to provide orientation to the Member States on the acceptability of cereals and cereal products and compound feed for animal feeding and to avoid disparities in the values accepted by the different Member States and the consequent risk of distortion of competition. In response to these consideration, further investigation and coordinated control programmes by member States were recommended and, finally, in 2013 the European Commission issued another recommendation (Commission Recommendation n 165 of 27 March 2013 on the presence of T-2 and HT-2 toxin in cereals and cereal products). This recommendation, concerning both the food and feed chain, give information to obtain once again more data on T-2 and HT-2 in cereals and cereal products. Recommendation concern first of all the sampling procedure applied by the food business operator, that might deviate from the provisions of Regulation (EC) No 401/2006 but should be representative for the lot sampled. Secondly, the recommendation gives performance criteria for the analysis of T-2 and HT-2 toxins. Finally, in the annex are summarized the Indicative levels for cereals and cereals products from which onwards/above which investigations should be performed (Table 2).

Table 2. Indicative levels for cereals and cereals products (*) (**)

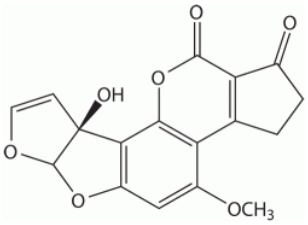
	Indicative levels for the sum of T-2 and HT-2 (μg/kg) from which onwards/above which investigations should be performed, certainly in case of repetitive findings (*)
1. **Unprocessed cereals** (***)	
1.1. barley (including malting barley) and maize	200
1.2. oats (with husk)	1000
1.3. wheat, rye and other cereals	100
2. **Cereal grains for direct human consumption** (****)	
2.1. oats	200
2.2. maize	100
2.3. other cereals	50
3. **Cereal products for human consumption**	
3.1. oat bran and flaked oats	200
3.2. cereal bran except oat bran, oat milling products other than oat bran and flaked oats, and maize milling products	100
3.3. other cereal milling products	50
3.4. breakfast cereals including formed cereal flakes	75
3.5. bread (including small bakery wares), pastries, biscuits, cereal snacks, pasta	25
3.6. cereal-based foods for infants and young children	15
4. **Cereal products for feed and compound feed** (*****)	
4.1. oat milling products (husks)	2000
4.2. other cereal products	100
4.3. compound feed, with the exception of feed for cats	250

(*) The levels referred to in this Annex are indicative levels above which, certainly in the case of repetitive findings, investigations should be performed on the factors leading to the presence of T-2 and HT-2 toxin or on the effects of feed and food processing. The indicative levels are based on the occurrence data available in the EFSA database as presented in the EFSA opinion. The indicative levels are not feed and food safety levels.
(**) For the purpose of this Recommendation rice is not included in cereals and rice products are not included in cereal products.
(***) Unprocessed cereals are cereals which have not undergone any physical or thermal treatment other than drying, cleaning and sorting.
(****) Cereal grains for direct human consumption are cereal grains which have undergone drying, cleaning, de-husking and sorting processes and on which no further cleaning and sorting processes will be performed before their further processing in the food chain.
(*****) The indicative levels for cereals and cereal products intended for feed and compound feed are relative to a feed with a moisture content of 12 %.

0.1.4 The aflatoxin M_1, toxicity, legislation and occurrence in milk derivative

Aflatoxins are a group of structurally related heterocyclic compounds of the group of mycotoxins produced by certain species of filamentous fungi of the genus *Aspergillus*. As *Aspergillus* species are capable of growing on a variety of substrates and under a variety of environmental conditions, they can contaminate a variety of food and feed commodities (Rustom, 1997). It has been shown that aflatoxin B1 (AFB_1) is the most potent hepatocarcinogen of this group of mycotoxins, aflatoxin M1 (AFM_1) is the 4-hydroxylated metabolite of AFB_1, it is a difuranocoumarin compound (figure 1) and is secreted in the milk of mammals that have consumed feeds contaminated by AFB_1 so that is known as "milk toxin".

Figure 1. Chemical Structure of aflatoxin M1



AFM_1 is also a hepatocarcinogen and is classified in Group 1 as carcinogenic to humans by the International Agency for Research on Cancer (IARC, 2000), although the carcinogenicity of AFM_1 is about ten times less than that of its precursor AFB_1. The potential hazard of aflatoxin in milk and milk products is considered to pose certain hygienic risks for human health, this fact has led to worldwide monitoring programs for the toxin in various commodities as well as regulatory actions by nearly all countries.

According to worldwide regulations for mycotoxins in food and feed compiled by the Food and Agriculture Organization of the United Nations, 60 countries have already established regulatory limits for AFM_1 in raw milk and milk products. The report also indicates that the limits vary from ND (not detectable) to 15 μg/kg (FAO, 2004). The values of 0.05 μg/kg and 0.5 μg/kg are the two most prevalent regulatory limits for AFM_1 in milk products, enforced in 34 and 22 countries, respectively. For example, the U.S. regulatory standard for AFM_1 is 0.5 μg/kg (FAO, 2004).

Regulatory limits seem to be a practical compromise between the need to have carcinogenfree commodities and the economic consequences of setting regulatory limits (Pohland and Yess, 1992), so that in all the developed countries the limit is stricter than the maximum recommended level of 500 ng/kg in raw milk set by the Codex Alimentarius (Codex Alimentarius, 2001).

The law that regulates the quality of milk products in Europe is the Commission Regulation (EU) 165/2010 amending regulation (EC) 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins. This regulation set the maximum levels of raw milk, heat-treated milk and milk for the manufacture of milk-based products at 0.050 μg/kg.

The maximum permitted level for AFM_1 established by the European Community is 0.025 μg/kg for infant formulae and follow-on formulae, including infant milk and follow-on milk, while the limit for raw milk and heat-treated milk is 0.05 μg/kg (EC, 2006). There are instead still several countries that have not yet established regulatory limits for AFM_1 in dairy products.

This limit is consistent with the PMTDI (provisional maximum tolerable daily intake), which is extimated at 0.2 ng/kg body weight *per die* (10-20 ng), so that, admitting 0.5 kg as daily milk intake for human consumption, it is tolarable a contamination of 20-40 ng/kg (10-20 ng/ 0.5 kg).

The occurrence of aflatoxin M_1 in cheese is due most of all to the presence of the toxin in raw milk or powdered milk used for cheese production, whereas synthesis of aflatoxins by fungi that can grow on cheese is negligible because the low level of carbohydrate does not make it a very suitable substrate. When cheese-making is carried out using AFM_1-contaminated milk, this toxin is likely to have become enriched in the final curd compared to that found in milk. This could be explained by both the capacity of AFM_1 to somehow bind caseins (Applebaum et al., 1982) and increased dry matter content (Deveci, 2007). AFM_1 is relatively stable during pasteurization, sterilization, preparation, ripening and storage of various dairy products. The consumption of cheese is widespread in Italy. For this purpose, determining the presence and levels of AFM_1 in cheese is required in the frame of the official control.

Therefore, it is necessary to note whether AFM_1 is present in final products like cheese because its concentration in them has been reported to be around 2.1-4.5 times higher than in the original milk used, depending on the cheese type (Van Egmond, 1983; Wiseman et al., 1983; Blanco et al., 1988; Deveci, 2007). In the EU, legal limits for AFM_1 in milk derivative lack, so in Italy it as been defined an "Enrichment Factor" (EF) for cheeses and other milk products, which is the mutual factor of the dairy yield (Annex 2 of DM Politiche Agricole e Forestali, 2003).

0.1.5 The cyanobacteria

Cyanobacteria, improperly called blue-green algae (BGA) are microscopic organisms that grow in wet environment throughout the world. They can be considered as simple aquatic plants that occur naturally in habitats such as rivers, lakes, damp soil, tree trunks, hot springs and snow. In external appearance and requirements for light, nutrients and carbon dioxide, they are similar to algae, but BGA are actually types of bacteria known as cyanobacteria.
They can vary considerably in shape, size and color, normally looking green and sometimes turning bluish when scums are dying.
Several types of cyanobacteria, like for example *Anabaena flos-aquae*, have gas-filled cavities that allow them to float to the surface or to different levels below the surface, depending on light conditions and nutrient levels. This causes the cyanobacteria to concentrate on the water surface, causing the typical pea-soup green color or blue-green "scum." Some cyanobacteria like *Planktothrix agardhii*, can be found in bottom sediments and float to the surface when mobilized by storm events and other sediment disturbances. Other cyanobacteria blooms may remain dispersed through the water column (Cylindrospermopsis sp.) leading to a generalized discoloration of the water.
With a worldwide distribution in both aquatic and terrestrial environments, BGA can be found in a wide range of ecosystems from the cold Antarctica to the hot springs of Japan, New Zealand and Italy (Papke et al.2003; Jungblut et al. 2005). There are more than 1500 known species of BGA.
When conditions are optimal, including light and temperature, levels of nutrients (i.e., phosphorous and nitrogen, and the ratio of the two), and lack of water turbulence, BGA can quickly multiply into a bloom. Although bloom conditions are likely to occur more often in warmer months especially during the late summer, the interrelationship of several factors causes large seasonal and year-to-year fluctuations in the cyanobacteria levels. Some strains can occur early in the summer season while others are only found during late summer. These phenomena can also be a consequence of euthrophication, i.e. a natural process that occurs in a body of water when it gradually builds up its concentration of plant nutrients.
In nature, this is a common phenomenon in freshwater ecosystems and is really a part of the normal aging process of many lakes and ponds. Some never experience it because of a lack of warmth and light, but many do. Over time, these bodies of freshwater change in terms of how productive or fertile they are. While this is different for each lake or pond, those that are naturally fed rich nutrients from a stream or river or some other natural source are described as "eutrophic," meaning they are nutrient-rich and therefore abundant in plant and animal life. Eutrophication is not necessarily harmful or bad, and the word itself is often translated from the Greek as meaning "well noutrished" or "good food." Cultural or artificial eutrophication occurs when human activity introduces increased amounts of nutrients, which speed up plant growth (including BGA) and eventually choke the lake of all of its animal life. Natural eutrophication is not rare, but usually a fairly slow and gradual process, occurring over a period of many centuries. It occurs naturally when for some reason, production and consumption within the lake do not cancel each other out and the lake slowly becomes overfertilized. Instead, artificial or human-caused eutrophication has become so common that the word eutrophication by itself has come to mean a very harmful increase and acceleration of nutrients. Human activities almost always result in the creation of waste, and many of these waste products often contain nitrates and phosphates, which are component of detergents and chemical fertilizers. Both nitrates and phosphates are absorbed by plants and are needed for growth. When they are washed into our lakes and ponds in a sufficient quantity, they act like fertilizer for plants and algae and speed up their rate of growth.

0.1.6 The cyanobacteria toxins

Cyanobacterial blooms can be harmful to the environment, animals, and human health not only because the bloom decay consumes oxygen, creating hypoxic conditions which result in plant and animal die-off, but also, although simple in morphology and nature, under favorable conditions of light and nutrients, some species of these prokaryotic organisms produce harmful secondary metabolites, known as cyanotoxins. These substances can have toxic effects on tissues, cells or organisms (Carmichael, 1992).
The conditions that cause cyanobacteria to produce cyanotoxins are not well understood. Some species with the ability to produce toxins may not produce them under all conditions. These species are often members of the common bloom-forming genera. Both non-toxic and toxic varieties of most of the common toxin-producing cyanobacteria exist, and it is impossible to tell if a species is toxic or not toxic by looking at it. Also, even when toxin-producing cyanobacteria are present, they may not actually produce toxins. Furthermore, some species of cyanobacteria can produce multiple types and variants of cyanotoxins. Molecular tests are available to determine if the cyanobacteria carry the toxin gene; quantitative cyanotoxin analysis is needed to determine if the cyanobacteria are actually producing the toxin.
In most cases, the cyanobacteria toxins exist intracellularly in the cytoplasm and are retained within the cell; for those species, when the cell dies or breaks, the cell membrane ruptures and the toxins are released into the water (extracellular toxins). However, in other species, cylindrospermopsin for example, a significant amount of the toxin may be naturally released to the water by the live cyanobacterial cell; the reported ratio is about 50% intracellular and 50% extracellular.

Cyanotoxin contamination may pose a serious risk to aquatic ecosystem and its users, they have been reported in both freshwater and marine environments with implications in human and animal poisonings (Dittmann and Wiegand 2006). Therefore, they need to be monitored to minimize their risks to both water quality and public health. In this regard, several countries have developed regulations or guidelines for cyanotoxins and cyanobacteria in drinking waters and, in some cases, in water used for recreation and agriculture (Burch 2006).
Toxins produced by cyanobacteria are classified after their effects on health as hepatotoxins (microcystins and nodularins), neurotoxins (anatoxins and saxitoxins), cytotoxins (cylindrospermopsin) and dermotoxins (Carmichael and Li 2006). Their production can be found in a diverse range of species. As to chemical, cyanobacteria are capable of producing two kinds of toxin, the cyclic peptides group and the alkaloids group.
In this study we want to focuse our attention on the most toxic cyanobacterial toxins so far found, the microcystins (MCCs), belonging to the chemical group of cyclic peptides and on cylindrospermopsin (CYN), an alkaloid compound recently studied (Falconer and Humpage 2005).

0.1.7 Microcystin producing organisms

Microcystins are a family of toxins with worldwide distribution (found in all of the main five continents), which are produced by different species of freshwater cyanobacteria, namely *Microcystis* (order Chroococcales), *Anabaena* (order Nostocales), and *Oscillatoria* (order Oscillatoriales).
The first one identified (Carmichael, 1994) was MC-LR, after, up to 90 MCCs have been isolated and characterised worldwide (Ferranti et al. 2009).
Biosynthesis of these compounds is performed by a family of multi-enzymatic complexes called nonribosomal peptide synthetases and polyketide synthases organized into repeated functional units known as modules. Its transcription and translation is independent of the messenger RNA and are organized in the genome into gene clusters. These encode enzymes for the biosynthesis, regulation, and export of the toxins. The first biosynthetic pathway to be sequenced was that of microcystins (Tillett et al., 2000). The *mcy* gene cluster assembly consists of 10 bidirectionally arranged genes that reside in two operons (*mcy A-C* and *mcy D-J*) of *M. aeruginosa*. The activities of these chromosomal gene products are primarily peptide synthetases [*mcy A-C*, *E*,*G*], polyketide synthases [*mcy D*, parts of *E* and *G*], and methylation [*mcy J*], epimerization [*mcy F*], dehydration [*mcy I*], and localization [*mcy H*], resulting in nonribosomal toxin synthesis. Disruption of some of these genes [*mcy A,B,D*, or *E*] resulted in no detectable toxin production (Nishiwaza et al., 2000).

0.1.8 Cylindrospermopsin producing organisms

CYN has been a subject of study since 1979 where it was first reported, with the increased number of publications on the detection of this toxin in several countries its occurrence has been reported either in environmental samples (water) or in isolated species so far in four of the five continents.
Cylindrospermopsis raciborskii was the first species reported as a producer of CYN. Other cyanobacteria species have been identified as CYN producers, namely *Umezakia natans* (Harada et al. 1994), *Aphanizomenon ovalisporum* (Banker et al. 1997), *Anabaena bergii* (Schembri et al. 2001), *Raphidiopsis curvata* (Li et al. 2001), *Aphanizomenon flos-aquae* (Preußel et al. 2006), *Anabaena lapponica* (Spoof et al. 2006) and more recently *Lyngbya wollei* (Seifert et al., 2007), clearly indicating that its production is not species specific. A summary of each report is presented in the following table (table 3):

Table 3. Cylindrospermopsin producing organism and occurrence worldwide

Continent	Species	Country
Europe	*Aphanizomenon ovalisporum*	Spain
	Anabaena lapponica	Finland
	Aphanizomenon flos-aquae	Germany
Asia	*Cylindrospermopsis raciborskii*	Thailand
	Umezakia natans	Japan
	Aphanizomenon ovalisporum	Israel
	Raphidiopsis curvata	China
America	*Aphanizomenon ovalisporum*	USA
Oceania	*Cylindrospermopsis raciborskii*	Australia
	Aphanizomenon ovalisporum	Australia
	Anabaena bergii	Australia
	Lyngbya wollei	Australia

It is interesting to point out that the *Cylindrospermopsis raciborskii* population from Brazilian freshwater is known to produce saxitoxin derivatives while CYN, which is commonly detected and isolated from Australia and Asia continents, has so far not been detected in South American strains.
Actually, cyanobacteria need a genetic cluster named *cyr* to produce CYN (Welker, von Döhren, 2006).
However, a recent investigation by Hoff-Risseti et al. (2013) reported for the first time the presence in *Cylindrospermopsis raciborskii* population from Brazilian freshwater nucleotidic sequences that are phylogenetically homologous to the complete clusters of *Cylindrospermopsis raciborskii* strains that produce the toxin.
All the four strains studied by Hoff-Risseti et al. had not the complete genetic cluster *cyr*, so that they were not able to biosyntetize CYN, but all of them were able to produce saxitoxin derivatives.
This discovery suggested a shift in the type of cyanotoxin production over time of South American strains of *Cylindrospermopsis raciborskii* and contributed to the reconstruction of the evolutionary history and diversification of cyanobacterial toxins.

0.1.9 Microcystins occurrence in the environment

Numerous outbreaks of human illness attributed to microcystin exposure are reported in the literature, e.g. from Australia, Brazil, USA, Africa, China and Europe**.** Several example are reported.
The first published report of an incidence of cyanobacteria poisoning dates from the poisoning of an Australian lake in 1878, these incidences have been attributed to cyanobacteria and, probably, to the toxic compound microcystin-LR (Francis et al., 1878).
There are no reports of human deaths known to have been specifically caused by microcystin-LR, although there are reports of health effects after exposure and there have been deaths attributed to microcystins in general. One of the most outstanding reports was an outbreak in Caruaru, Brazil, in 1996. 116 patients experienced multiple effects: visual disturbance, nausea, vomiting and muscle weakness. One hundred developed acute liver failure and 52 suffered from symptoms of what is now called "Caruaru Syndrome". The syndrome was caused by dialysis therapy with water that had not been properly treated. (Pouria et al, 1998).
An interesting case concerning the USA is that of Upper Klamath Lake in southern Oregon, from whose waters *Aphanizomenon flos-aquae* is harvested for the production of BGA dietary supplements. In this lake *Microcystis aeruginosa* is a regular occurrence, coexisting with *A. flos-aquae*, it can be collected inadvertently during the harvesting process, resulting in microcystin contamination of BGA products (Gilroy et al., 2000).
As to Africa, one of the most recent report revealed the presence of MC-LR, MC-YR and MC-RR in the he Hartbeespoort Dam (South Africa's North-West Province), a reservoir used for agricultural, domestic supply of raw potable water and recreational activities, where eutrophication and cyanobacterial blooms have long been a cause of water-quality problems in this reservoir (Elbert et al., 2012).
As to Asia, they have been reported several studies regarding the occurrence of MCCs in lakes of China (Sakai et al. 2013, Wang et al. 2013) and other countries of the south-east (Welker et al., 2005).
In Europe, the presence of MCCs producing BGA of *Planktothrix* genus has widely been studied (Kurmayer et al, 2004) in Germany, Austria, France and Switzerland, while in our laboratories, a novel variant of MCCs was found in Averno Lake (Ferranti et al., 2009).
The health risk for humans is due not only to direct exposure, but MCCs can also accumulate in tissues of aquatic organisms and transfer to other animals and to humans through the food chain (De Magalhaes et al., 2001). Also, a large number of cases of animal deaths (domestic, livestock and wildlife) after drinking water with cyanobacterial scums was reported (Zimba et al. 2001).

0.1.10 Cylindrospermopsin occurence in the environment

The first description of CYN occurred after an outbreak of hepatoenteritis in Palm Island, northern Queensland, Australia, in 1979 (Bourke et al. 1983). This was the first documented case of this cyanotoxin affecting 148 people, mostly children, which required hospitalization and presented symptoms of gastroenteritis (Byth 1980).
Cylindrospermopsis raciborskii was the dominant species in the Solomon dam after this incident. This species was reported as nontoxic until this incident and known to form blooms in tropical environments (Hawkins et al.1985).
Later, Ohtani et al. (1992) characterized for the first time the chemical structure of this cyanotoxin from a *C. raciborskii* strain isolated from the Palm Island incident and named it after its given genus. Since then, it has been described in other cyanobacteria species all belonging to the *Nostocales* Order with two exceptions *Umezakia natans* (Order Stigonematales) as previously descrive by Harada et al. (1994) and more recently *Lyngbya wollei* (Order Oscillatoriales) by Seifert et al. (2007).
In Australia, documented reports on the presence of CYN in water samples are mainly attributed to the occurrence of *C. raciborskii* blooms, the first species where this toxin was described and that constitutes in this country a major concern (Eaglesham et al. 1999).

Saker et al. first described the presence of CYN in a field sample in the Oceania continent (Saker et al., 1999a) where two distinct morphotypes of CYN-producing *Cylindrospermopsin raciborskii* from the Solomon dam water system in February of 1996 were isolated. Another study is also from Saker et al. (Saker et al.1999b) where CYN-producing *Cylindrospermopsin raciborskii* from the water of a farm dam was isolated to which it was attributed the death of the cattle that drank from that water. Further evidence of the presence of CYN in Australian freshwaters were by Fabbro et al. (2001) and in New Zealand (Stirling & Quilliam 2001).
In Europe, the presence of CYN was for the first time reported by Fastner et al. (2003); the toxin was found in two German lakes (Melangsee and Langer See); however, CYN production was not attributed to any of the *Cylindrospermopsis raciborskii* isolates.
Later, Fastner et al. (2007) found its presence in 63 lakes from Germany showing its high prevalence in this country with concentrations reaching up to 12 µg/l.
CYN was detected in a Spanish reservoir (Quesada et al. 2006), where it was associated with a bloom of *Aphanizomenon ovalisporum*.
CYN was then found in many others european country, including Italy, where its presence in the water samples of Albano Lake was assigned to *Cylindrospermopsis raciborskii* (Manti et al., 2005); in our laboratories CYN has been recently detected and quantified from the waters of Averno Lake without assigning its production to a particular species (Gallo et al., 2009).

0.1.11 Toxicological data of MCCs

There have been many reports of the intoxication of birds, fish and other animals by cyanobacteria toxins (Vasconceles et al., 2001; Alonso-Andicoberry et al., 2002; Best et al., 2002; Romanowska-Duda et al., 2002; Krienitz et al., 2003). As stated before, blooms of cyanobacteria usually follow enrichment by nutrients such as phosphates and nitrates in the water. Most of these nutrients are derived from human wastes such as sewage and detergents, industrial pollution, run-off of fertilizers from agricultural land, and the input of animal or bird wastes from intensive farming (Bell & Codd, 1994; Baker, 2002). Illnesses caused by cyanobacterial toxins to humans fall into three categories; gastroenteritis and related diseases, allergic and irritation reaction, and liver diseases (Bell & Codd, 1994). Microcystins have also been implicated as tumour-promoting substances (An & Carmichael, 1994; Bell & Codd, 1994; Rudolph–Böhner et al., 1994; Trogen et al., 1996; Zegura et al., 2003). The LD_{50} of microcystin–LR i.p or i.v. in mice and rats is in the range 36-122 µg/kg, while the inhalation toxicity in mice is similar; LCT_{50}=180 mg/min/m3 or LD_{50}=43 µg/kg (Stoner et al., 1991). Therefore microcystin-LR has comparable toxicity to chemical organophosphate nerve agents. Symptoms associated with microcystin intoxication are diarrhea, vomiting, piloerection, weakness and pallor (Bell & Codd, 1994). Microcystin targets the liver, causing cytoskeletal damage, necrosis and pooling of blood in the liver, with a consequent large increase in liver weight. Membrane blebbing and blistering of hepatocytes *in vitro* has been observed (Runnegar et al., 1991; Romanowska-Duda et al., 2002). High chromatin condensation and apoptotic bodies were observed in 90% of the cells of *Sirodela oligorrhizza* and rat hepatocytes after a treatment with microcystin-LR (MC-LR=500mug/dm) (Romanowska-Duda et al., 2002). Death appears to be the result of haemorrhagic shock (Hermansky et al., 1990) and can occur within a few hours after a high dose of microcystin-LR (Falconer et al., 1981; Bell & Codd, 1994). The concentration of microcystin–LR in drinking water for humans as prescribed by the world health organization (WHO) is 1 µg/L (WHO, 1998), however, Ueno and coworkers (1996) proposed a value of 0.01 µg/L, based on a possible correlation of primary liver cancer in certain areas of China with the presence of microcystins in water of ponds, rivers and shallow wells. The Italian law set 0.84 µg/L total MCCs as the tolerable limit in freshwaters (Italian Ministry of Health, 1998), to prevent possible intoxication as a consequence of swimming and recreaction activities.
Neurotoxic effects and an involvement of MCC exposure in the onset and exacerbation of human neurodegenerative diseases are currently under debate and cannot be ruled out (Li et al., 2012).
It is thus crucial to emphasize that current TDI (i.e. 0.04 µg/kg body weight) calculations do not include the potential neurotoxic effects. Moreover, the whole MCC risk assessment is based on the toxicological dataset for one MCC congener, MC-LR only (Gilroy et al., 2000). However, there are ≥90 MCC congeners, some of which may have an overall greater toxicity than MC-LR (Dietrich et al., 2008).

0.1.12 Toxicological data of CYN

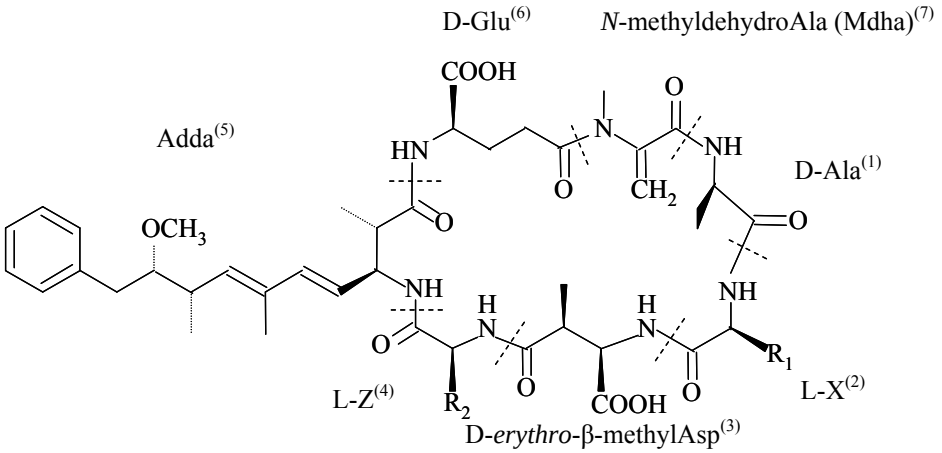
CYN is cytotoxic, inhibiting protein synthesis mechanisms (Froscio et al. 2001, 2008, Humpage et al. 2005); toxic effects on the main target tissues, that is liver, kidney, thymus and lung have been also descrive (Terao et al., 1994). Moreover, *in vitro* mutagenicity (Shaw et al., 2000), carcinogenity (Humpage et al., 2000 Shen et al., 2002) and genotoxic activities (Bain et al., 2007).
A NOEL (*No observed effect level*) value at 30 µg/kg body weight per die was evaluated for CYN (Humpage &Falconer, 2003), from which a provisional TDI (*tolerable daily intake*) of 0.03 µg/kg body weight per die can be derived. Further data regarding CYN distribution in freshwaters and human absorption mechanism are necessary; in the meantime, a guide value (GV) at 1 µg/L in drinking water was proposed (Codd et al., 2005).

After this guideline was proposed, several nongovernmental agencies, such as research institutions, assumed this value for toxicity assessment in water quality management and control. Later, few governments adopted this guideline value for CYN in drinking water, namely Brazil and New Zealand (Burch 2006). No guideline values for CYN in recreation and bathing waters have been adopted yet. Although these two countries have recommended values for CYN in drinking water, the established values differ greatly. While in Brazil the federal legislation includes a guideline value for CYN of 15 µg/L, in New Zealand, the maximum acceptable value is of 1 µg/L (Burch 2006).

0.1.13 Chemical characterization of MCCs

The general structure of microcystins is cyclo(D-Ala(1)-L-X(2)—DMeAsp(3)-L-Z(4)-Adda(5)-D-Glu(6)-Mdha(7)). L-X and L-Z in position 2 and 4 of the ring are variable L-amino acids, D-MeAsp is D-erythromethylaspartic acid, Mdha is *N*-methyldehydroalanine, and Adda moiety is (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid. Each microsystin is named depending on the identity of X and Z amino acids. There are over 50 different microcystins that differ primarily in the two L-amino acids at positions 2 and 4, and methylation/demethylation on MeAsp and MDha. The unusual amino acid Adda is essential for the expression of biological activity. Other microcystins are characterized largely by variation in the degree of methylation; amino acid 3 has been found to be D–aspartic acid, replacing β–methylaspartic acid and amino acid 7 to be dehydroalanine, replacing N–methyldehydroalanine (An and Carmichael, 1994; Trogen et al., 1996). The most common microcystin, is microcystin–LR, where the variable L–amino acids are leucine (L) and argenine (R). The general structure is shown in Figure 2. Some esters of glutamic acid have been observed for amino acid 6 replacing γ-linked glutamic acid itself and N-methylserine sometimes replaces amino acid 7. Variations in the Adda subunit (amino acid 5) include 0-acetyl-0-demethyl-Adda and (6Z)-Adda (Rinehart et al., 1988). However, the Adda molecule's overall shape seems to be critical since the (6Z0-Adda)(*cis*) isomer is inactive (Rinehart et al., 1988). The adda and D-glutamic acid portions of the microcystin-LR molecule play highly important roles in the hepatoxicity of microcystins. Esterification of the free carboxyl group of glutamic acid results essentially in inactive compounds. Some of the Adda subunits assert little effect, especially the 0-dimethyl-0-acetyl analogs.

Figure 2. The general molecular structure of a microcystin.



0.1.14 Chemical characterization of CYN and analogues

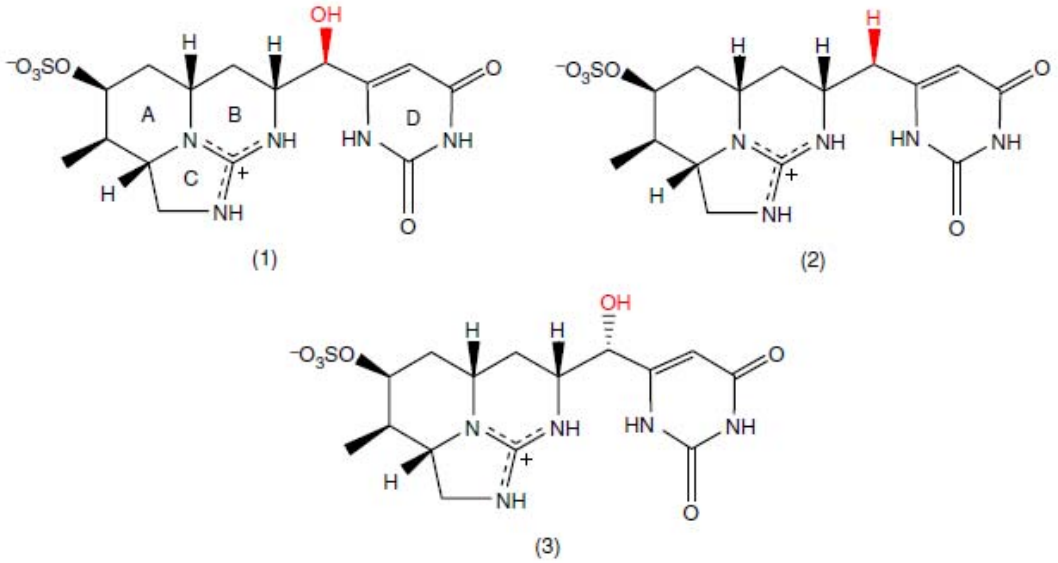
In New Zealand, the first report was from a scum that formed on the surface of the recreational Lake Waitawa near Wellington. The sample was tested for the presence of CYN by LC-MS analysis but not quantified due to the lack of standard for the assay (Stirling & Quilliam 2001).

CYN is an alkaloid, ($[C_{15}H_{21}N_5O_7S]$; 415.43 g/mol, glassy solid), a sulfate ester of a tricyclic guanidine moiety (rings A, B & C), with a uracil ring (D) (1) and its zwitterionic nature makes it a highly water-soluble molecule.

The gross structure of CYN was solved by mass spectroscopy (MS) and nuclear magnetic resonance (NMR), first by Ohtani et al. (1992). However, it was found later an incorrect feature in the first identification, the orientation of the hydroxyl group whose epimer was later identified by Banker et al. (2000) as 7-epicylindrospermopsin (3), a toxic minor metabolite of *Aphanizomenon ovalisporum*.

Norris et al. in 1999 were able to isolate CYN and the analogue 7-deoxy-cylindrospermopsin (2) by solidphase extraction (SPE) followed by semi-preparative chromatography.

Figure 3. The molecular structure of cylindrospermopsin (1), 7-deoxy-cylindrospermopsin (2) and 7-epicylindrospermopsin (3).



Concerning the stability of the molecule, Chiswell et al. (1999) found that CYN is a very stable compound showing no degradation at boiling temperature (100°C for 5 min); when exposed to pH changes between 4 and 10 for a period of 8 weeks, it only degrades 25%, and in the solid state or in pure aqueous solutions, CYN does not show any degradation. However, while in culture exposed to sunlight, it decomposes rapidly (half-life 1-5 h). CYN degradation via chlorination occurs immediately at pH 6, but the molecule is more stable to this water treatment process at lower values of pH (Senogles et al. 2000).

0.1.15 Legal aspects of cyanobacterial products

Few regulations govern the use of algal and cyanobacterial products. In Australia and New Zealand, the Food Standards Australia New Zealand agency regulates the use of novel food ingredients, such as products from microalgae. In Canada, BGA products are sold as food. Health Canada has screened cyanobacterial products available on the Canadian market for the presence of MCCs, and the results showed that the toxins were detected in non-Spirulina products. In the United States, the FDA regulates the safety of food, including algal products which are classified as "other dietary supplements". To date none of the beneficial effects could be scientifically and clinically confirmed. Moreover, the FDA mandated that companies selling cyanobacterial dietary supplements clearly state that these products have no pharmaceutical or curative capabilities: "*This product is not intended to diagnose, treat, cure or prevent any disease*".

Placing an algal product as a novel food on the market within the European Community is regulated by the European Parliament and the Council of the European Union (Regulation (EC) No. 258/97), and companies are required to submit their application in accordance with Commission Recommendation 97/618/EC.

Issues of food safety at the international level are dealt with by the Codex Alimentarius Commission, which was created by the Food and Agriculture Organization (FAO) and the WHO with the aim of developing food standards and guidelines. This Commission rejected the proposal that microalgae should be regarded as food because of the lack of history on their safe use (Codex Alimentarius Commission, 2003).

Figure 4. Algae dietary supplement in different formulations

0.1.16 Health concerns related to cyanobacterial products consumption

Several acute adverse health effects such as nausea, vomiting, diarrhea, constipation and upset stomach were reported by consumers of BGA products (Braun and Cohen, 2010). Allergic reactions including asthmatic wheezing, hay fever or conjunctivitis and skin irritations have also been reported as have drug interactions with antihistamines, blood-thinners and diabetes medications. Several case reports exist that appear to corroborate the health risks associated with consumption of algae dietary supplements. Acute rhabdomyolysis was reported in a 28-year old man following ingestion of Spirulina supplements for one month (Mazokopakis et al., 2008), generalized seizures associated with hypercalcemia of a day-old baby was found to be related to the mother's long-term consumption of Spirulina supplements (Moulis et al., 2012) and anaphylaxis was reported in a 14-year-old adolescent who had previously experienced urticaria, labial oedema and asthma 6 h after consumption of five Spirulina tablets (Petrus et al., 2010).

To date, the cytotoxic components in the dietary supplement extracts responsible for the effects observed have not been identified, albeit it is known that algae readily absorb heavy metals e.g. lead or mercury and can result in contamination levels in the harvested products in the range of μg/g (Fugh-Berman, 2003).

0.2 REFERENCES

Alonso-Andicoberry C, Garcia-Villada L, Lopez-Rodas V, Costas E (2002) Catastrophic mortality of flamingos in a Spanish National park caused by cyanobacteria. Veterinary Record 151:706-707.

An J, Carmichael WW (1994). Use of a colorimetric protein phosphatase inhibition assay and enzyme linked immunosorbent assay for the study of microcystins and nodularins. Toxicon 32:1495-1507.

Applebaum RS, Brackett RE, Wiseman DW, Marth EH (1982) Aflatoxins: toxicity to dairy cattle and occurrence in milk and milk products. Journal of Food Protection 45:752-777.

Bain P, Shaw G, Patel B (2007) Induction of p53-regulated gene expression in human cell lines exposed to the cyanobacterial toxin cylindrospermopsin. J Toxicol Environ Health Part A 70:1687-1693.

Baker LC (2002) Cyanobacterial harmful algal blooms (CyanoHABs): Developing a public health response. Lake and Reservoir Management 18:20-31.

Banker R, Carmeli S, Hadas O, Teltsch B, Porat R, Sukenik A (1997) Identification of cylindrospermopsin in *Aphanizomenon ovalisporum* isolated from Lake Kinneret, Israel. J Phycol. 33:613–616.

Banker R, Teltsch B, Sukenik A, Carmeli S (2000) 7-Epicylindrospermopsin, a toxic minor metabolite of the cyanobacterium *Aphanizomenon ovalisporum* from Lake Kinneret, Israel. J Nat Prod 63:387–389.

Bell SG, Codd GA (1994). Cyanobacterial toxins and human health. Rev. Med. Microbiol. 5:256-264.

Best JH, Pflugmacher S, Wiegand C, Eddy FB, Metcalf JS, Codd GA (2002) Effects on enteric bacterial and cyanobacterial lipopolysaccharides, and of microcystin-LR, on glutathione Stransferase activities in zebra fish (*Danio rerio*). Aquatic Toxicol. 60:223-231.

Blanco JL, Domingues L, Gomes-Lucia E, Garayzabal JFF, Goyache J, Suarez J (1988) Behavior of aflatoxin during the manufacture, ripening and storage of Manchego-type cheese. Journal of Food Science 53:1373-1376.

Bourke ATC, Hawes RB, Neilson A, Stallman ND (1983) An outbreak of hepato-enteritis (the Palm Island mystery disease) possibly caused by algal intoxication. Toxicon. 21:45–48.

Braun L, Cohen M. Herbs & Natural Supplements: An Evidence-based Guide. Churchill Livingstone 2010.

Burch MD. Effective doses, guidelines & regulations. In Proceedings of the Interagency, International Symposium on Cyanobacterial Harmful Algal Blooms (ISOC-HAB), State of the Science and Research Needs ed. Hudnell HK. New York, USA: Springer 2006 pp. 831–853.

Burch MD (2006) Effective doses, guidelines & regulations. In Proceedings of the Interagency, International Symposium on Cyanobacterial Harmful Algal Blooms (ISOC-HAB), State of the Science and Research Needs ed. Hudnell, H.K.. New York, USA: Springer. pp. 831–853.

Byth S (1980) Palm island mystery disease. Med J Aust 2:40–42.

Carmichael WW (1992) Cyanobacteria secondary metabolites – the cyanotoxins. J Appl Bacteriol 72:445–459.

Carmichael WW (1994) The toxin of cyanobacteria. American Science 270:78-86.

Carmichael WW, Li RH (2006) Cyanobacterial toxins in the Salton Sea. Saline Ecosyst 2:5.

Chiswell RK, Shaw GR, Eaglesham GK, Smith MJ, Norris RL, Seawright AA, Moore MR (1999) Stability of cylindrospermopsin, the toxin from the cyanobacterium *Cylindrospermopsis raciborskii*, effect of pH, temperature, and sunlight on decomposition. Environ Toxicol 14:155–165.

Codd GA, Morrison LF, Metcalf JS (2005) Cyanobacterial toxins: risk management for health protection. Toxicol Appl Pharmacol 203:264-72.

Codex Alimentarius Commissions (2001) Comments submitted on the draft maximum level for Aflatoxin M1 in milk. Codex committee on food additives and contaminants 33rd sessions. Hauge, The Netherlands.

Codex Alimentarius Commission (2003) Joint FAO/WHO Food Standards Programme, 4th Session, Yokohama, Japan, March 11–14, 2003.

Commission Recommendation 97/618/EC of 29 July 1997 concerning the scientific aspects and the presentation of information necessary to support applications for the placing on the market of novel foods and novel food ingredients and the preparation of initial assessment reports under Regulation (EC) No 258/97 of the European Parliament and of the Council.

Commission Recommendation 2002/214/EC of 12 March 2002 on the coordinated inspection programmes in the field of animal nutrition for the year 2002 in accordance with Council Directive 95/53/EC (OJ L 70, 13.3.2002, p. 20).

Commission Recommendation 2004/163/EC of 17 February 2004 on the coordinated inspection programme in the field of animal nutrition for the year 2004 in accordance with Council Directive 95/53/EC (OJ L 52, 21.2.2004, p. 70).

Commission Recommendation 2005/187/EC of 2 March 2005 on the coordinated inspection programme in the field of animal nutrition for the year 2005 in accordance with Council Directive 95/53/EC (OJ L 62, 9.3.2005, p. 22).
Commission Recommendation n 165 of 27 March 2013 on the presence of T-2 and HT-2 toxin in cereals and cereal products.

Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs.

Commission Regulation (EC) No 1881/2006 of 19 December 2006 Setting Maximum Levels for Certain Contaminants in Foodstuffs.

Commission Regulation (EU) No 165/2010 of 26 February 2010 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins

De Magalhaes VF, Moraes Soraes R, Azevedo SMFO (2001) Microcystin contamination in fish from the Japarecaguà Lagoon (Rio de Janeiro, Brazil): ecological implication and human health risk. Toxicon 39:1077-1085.

Desjardins AE. Fusarium Mycotoxins: Chemistry, Genetics, and Biology. American Phytopathological Society Press. St. Paul, MN, USA, 2006.

Deveci O (2007) Changes in the concentration of aflatoxin M1 during manufacture and storage of White Pickled cheese. Food Control 9:1103-1107.

Dietrich DR, Fischer A, Michel C, Hoeger SJ (2008) Toxin mixture in cyanobacterial blooms-a critical comparison of reality with current procedures employed in human health risk assessment. Adv Exp Med Biol 619:885-912.

Dittmann E, Wiegand C (2006) Cyanobacterial toxins – occurrence, biosynthesis and impact on human affairs. Mol Nutr Food Res 50:7–17.

Eaglesham GK, Norris RL, Shaw GR, Smith MJ, Chiswell RK, Davis BC, Neville GR, Seawright AA (1999) Use of HPLC-MS/MS to monitor cylindrospermopsin, a blue-green algal toxin, for public health purposes. Environ Toxicol 14:151–154.

Elbert A, Mbukwa, Titus AM, Msagati, Bhekie B, Mamba (2012) Quantitative Variations of Intracellular Microcystin-LR, -RR and -YR in Samples Collected from Four Locations in Hartbeespoort Dam in North West Province (South Africa) During the 2010/2011 Summer Season. Int J Environ Res Public Health 9(10): 3484–3505.

Fabbro L, Baker M, Duivenvoorden L, Pegg G, Shiel R (2001) The effects of the ciliate *Paramecium cf. caudatum* Ehrenberg on toxin producing Cylindrospermopsis isolated from the Fitzroy river, Australia. Environ Toxicol 16:489–497.

Falconer IR, Jackson ARB, Langley J, Runnegar MT (1981) Liver pathology in mice poisoning by the blue-green alga *Microcystis aeruginosa*. Aust. J. Biol. Sci. 24: 179-187.

Falconer IR, Humpage AR (2005) Health risk assessment of cyanobacterial (blue-green algal) toxins in drinking water. Int J Environ Res Public Health 2: 43–50.

FAO (2004) Worldwide Regulations for Mycotoxins in Food and Feed in 2003. Food and Nutrition Paper 81. Rome: FAO.

Fastner J, Heinze R, Humpage AR, Mischke U, Eaglesham GK, Chorus I (2003) Cylindrospermopsin occurrence in two German lakes and preliminary assessment of toxicity and toxin production of *Cylindrospermopsis raciborskii* (Cyanobacteria) isolates. Toxicon. 42:313–321.

Fastner J, Rücker J, Stüken A, Preußel K, Nixdorf B, Chorus I, Köhler A, Wiedner C (2007) Occurrence of the cyanobacterial toxin cylindrospermopsin in Northeast Germany. Environ Toxicol 22:26–32.

Ferranti P, Fabbrocino S, Nasi A, Caira S, Bruno M, Serpe L, Gallo P (2009) Liquid chromatography coupled to quadrupole time-of-flight tandem mass spectrometry for microcystin analysis in freshwaters: method performances and characterisation of a novel vatiant of microcystin-RR. Rapid Commun Mass Spectrom 23:1328-1336.

Francis G (1878) Poisonous Australian lake. Nature 18:11-12.

Froscio SM, Humpage AR, Burcham PC, Falconer IR (2001) Cell-free protein synthesis inhibition assay for the cyanobacterial toxin cylindrospermopsin. Environ Toxicol 16:408-412.

Froscio SM, Humpage AR, Wickramasinghe W, Shaw G, Falconer IR (2008) Interaction of the cyanobacterial toxin cylindrospermopsin with the eukaryotic protein synthesis system. Toxicon 51:191-198.
Fugh-Berman A. The 5-Minute Herb and Dietary Supplement Consult. Lippincott Williams & Wilkins. 2003

Gallo P, Fabbrocino S, Cerulo MG, Ferranti P, Bruno M, Serpe L (2009) Determination of cylindrospermopsin in freshwaters and fish tissue by liquid chromatography coupled to electrospray ion trap mass spectrometry Rapid Commun Mass Spectrom. 23: 3279–3284.

Gilroy DJ, Kauffman KW, Hall RA, Huang X, Chu FS (2000) Assessing potential health risks from microcystin toxins in blue-green algae dietary supplements. Environ Health Perspect 108(5):435–439.

Harada KI, Ohtani I, Iwamoto K, Suzuki M, Watanabe MF, Watanabe M, Terao K (1994) Isolation of cylindrospermopsin from a cyanobacterium Umezekia natans and its screening method. Toxicon 32:73–84.

Hermansky SJ, Casey PJ, Stohs SJ (1990). Cuclosporin A – a chemoprotectant against microcystin-LR toxicity. Toxicol. Lett. 54: 279-285.

Hoff-Risseti C, Dörr FA, Schaker PD, Pinto E, Werner VR, Fiore MF (2013) Cylindrospermopsin and Saxitoxin Synthetase Genes in *Cylindrospermopsis raciborskii* Strains from Brazilian Freshwater. PLoS One. Aug 28:8.

Hawkins PR, Runnegar MTC, Jackson ARB and Falconer IR (1985) Severe hepatotoxicity caused by the tropical cyanobacterium (blue-green alga) *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raju isolated form a domestic supply reservoir. Appl Environ Microbiol 50:1292–1295.

Humpage AR, Falconer IR (2003) Oral toxicity of the cyanobacterial toxin cylindrospermopsin in male Swiss albino mice: determination of no observed adverse effect level for deriving a drinking water guideline value. Environ Toxicol 18:94–103.

Humpage AR, Fenech M, Thomas P, Falconer IR (2000) Micronucleus induction and chromosome loss in transformed human white cells indicate clastogenic and aneugenic action of the cyanobacterial toxin, cylindrospermopsin. Mutation Research/DNA Repair 472:155-161.

Humpage AR, Fontaine F, Froscio S, Burcham P, Falconer IR (2005) Cylindrospermopsin genotoxicity and cytotoxicity : role of cytochrome P-450 and oxidative stress. Journal of Toxicology and Environmental Health, Part A 68:739-753.

IARC (2002) IARC Monographs on the Evaluation of Carcinogenic Risks to Humans,., Some Traditional Herbal Medicines, Some Mycotoxins, Naphthalene and Styrene. IARC Press. Lyon, France 82

Italian Ministry of Health (1998). Health criteria and other supporting information. Circolare Ministero della Sanità 31/7/1998 IX, 400,4/13,1/3/1447.

Li R, Carmichael WW, Brittain S, Eaglesham GK, Shaw GR, Liu Y, Watanabe MM (2001) First report of the cyanotoxins cylindrospermopsin and deoxycylindrospermopsin from *Raphidiopsis curvata.* J Phycol 37:1121–1126.

Joint FAO/WHO Expert Committee on Food Additives (JECFA). Safety Evaluation of Certain Mycotoxins in Food. Food and Agriculture Organisation. Rome, Italy, 2001.

Jungblut AD, Hawes I, Mountfort D, Hitzfeld B, Dietrich DR, Burns BP, Neilan BA (2005) Diversity within cyanobacterial mat communities in variable salinity meltwater ponds of McMurdo Ice Shelf., Antarctica. Environ Microbiol 7:519–529.

Königs M, Mulac D, Schwerdt G, Gekle M, Humpf HU (2009) Metabolism and cytotoxic effects of T-2 toxin and its metabolites on human cells in primary culture. Toxicology 258:106–115.

Krienitz L, Ballot A, Kotut K, Wiegand C, Putz S, Metcalf JS, Codd GA, Pflugmacher S (2003). Contribution of hot spring cyanobacteria to the mysterious deaths of Lesser flamingos at Lake Bogoria, Kenya. FEMS Microbiol. Ecol. 43: 141-148.

Kurmayer R, Christiansen G, Fastner Börner JT (2004) Abundance of active and inactive microcystingenotypes in populations of the toxic cyanobacterium *Planktothrix* spp. Environmental Microbiology.

Le Bars J, Le Bars P (1996) Recent acute and subacute mycotoxicoses recognized in France. Vet Res 27:383–394.

Li R, Carmichael WW, Brittain S, Eaglesham GK, Shaw GR, Liu Y, Watanabe MM (2001) First report of the cyanotoxins cylindrospermopsin and deoxycylindrospermopsin from Raphidiopsis curvata. J Phycol 37:1121–1126.

Manti G, Mattei D, Messineo V, Melchiorre S, Bogialli S, Sechi N, Casiddu P, Luglie' A, Di Brizio M, Bruno M (2005) First report of *Cylindrospermopsis raciborskii* in Italy Harmful Algae News 28: 8-9.
Mazokopakis E, Karefilakis C, Tsartsalis A, Milkas A, Ganotakis E (2008) Acute rhabdomyolysis caused by Spirulina (*Arthrospira platensis*). Phytomedicine 15:525–527.

Medentsev AG and Akimenko VK. Naphthoquinone metabolites of the fungi. Phytochemistry. Vol. 47, No.6 1998, pp. 935-959.

Ministero delle politiche agricole e forestali decreto 31 luglio 2003 Modalita' di attuazione della legge 30 maggio 2003, n. 119, concernente il prelievo supplementare nel settore del latte e dei prodotti lattiero-caseari. GU 183.

Moulis G, Batz A, Durrieu G, Viard C, Decramer S, Montastruc J (2012) Severe neo-natal hypercalcemia related to maternal exposure to nutritional supplement containing Spirulina. Eur J Clin Pharmacol 68:221–222.

Nishizawa T, Ucda A, Asayama M, Fujii K, Harada, Ochi K, Sharai M (2000) Polyketide syntase gene coupled to the peptide synthetase module involved in the biosynthesis of the cyclic heptapeptide microcystin. J Biochem 127:779-789.

Norris RL, Eaglesham GK, Pierens G, Shaw GR, Smith MJ, Chiswell RK, Seawright AA, Moore MR (1999) Deoxycylindrospermopsin, an analog of cylindrospermopsin from *Cylindrospermopsis raciborskii*. Environ Toxicol 14:163–165.

Ohtani I, Moore RE, Runnegar MTC (1992) Cylindrospermopsin, a potent hepatotoxin from the bluegreen alga *Cylindrospermopsis raciborskii*. J Am Chem Soc 114:7942–7944.

Papke RT, Ramsing NB, Bateson MM, Ward DM (2003) Geographical isolation in hot spring cyanobacteria. Environ Microbiol 5:650–659.

Petrus M, Culerrier R, Campistron M, Barre A, Rougé P (2010) First case report of anaphylaxis to spirulin: identification of phycocyanin as responsible allergen. Allergy 65:924–925.

Pittet A. Natural Occurrence of Mycotoxins in Foods and Feeds: a Decade in Review. In: Mycotoxins and Phycotoxins in Perspective at the Turn of the Millennium (Proceedings of the Xth International IUPAC Symposium

on Mycotoxins and Phycotoxins, 21–25 May, 2000, Guaruja, Brazil). Edited by de Koe WJ et al., Publ. de Koe WJ, Hazekamp 2, 6707 HG Wageningen (The Netherlands), 2001. Chapter 6, pp. 153–172.

Pohland AE, Yess NJ (1992) Food Contaminants: scientific and public health implications. Proceedings of the Nutrition Society of Australia17:1-12.

Pouria S, de Andrade A, Barbosa J, Cavalcanti RL, Barreto VT, Ward CJ, Preiser W, Poon GK, Neild GH, Codd GA (1998) Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. 4;352(9121):21-6.

Preußel K, Stüken A, Wiedner C, Chorus I, Fastner J (2006) First report on cylindrospermopsin producing *Aphanizomenon flos-aquae* (Cyanobacteria) isolated from two German lakes. Toxicon 47:156–162.

Quesada A, Moreno E, Carrasco D, Paniagua T, Wormer L, De Hoyos C, Sukenik A (2006) Toxicity of *Aphanizomenon ovalisporum* (Cyanobacteria) in a Spanish water reservoir. Eur J Phycol 41:39–45.

Richard JL, Payne GA. Mycotoxins: Risks in Plant, Animal and Human Systems. Council for Agricultural Science and Technology. Ames, IA, USA, 2002.

Rinehart KL, Harada K, Namikoshi M, Chen C, Harvis CA, Munro MHG, Blunt JW, Mulligan PE, Beasley VR, Dahlem AM, Carmichael WW (1988). Nodularin, microcystin, and configuration of Adda. J Am Chem Soc 110:8557-8558.

Romanowska-Duda Z, Mankiewicz J, Tarczynska M, Walter Z, Zalewski M (2002) The effect of toxic cyanobacteria (blue-green algae) on water plants and animal cells. Polish J. Environ. Studies 11: 561-566.

Rudolph-Böhner S, Wu J-T, Moroder L (1993). Identification and characterization of Microcystin-LY from Microcystis aeruginosa (Strain 298). Biol. Chem. 374: 635-640.

Runnegar MTC, Gerdes RG, Falconer IR (1991). The uptake of the cyanobacterial hepatoxin microcystin by isolated rat hepatocytes. Toxicon 29: 43-51.

Rustom IYS (1997) Aflatoxin in food and feed: occurrence, legislation and inactivation by physical methods. Food chemistry 59: 57-67.

Sakai H, Hao A, Iseri Y, Wang S, Kuba T, Zhang Z, Katayama H (2013) Occurrence and distribution of microcystins in Lake Taihu, China. ScientificWorldJournal 16:838176.

Saker ML, Neilan BA, Griffiths DJ (1999a) Two morphological forms of *Cylindrospermopsis raciborskii* (cyanobacteria) isolated from Solomon Dam, Palm Island, Queensland. J Phycol. 35:599–606.

Saker ML, Thomas AD, Norton JH (1999b) Cattle mortality attributed to the toxic cyanobacterium *Cylindrospermopsis raciborskii* in an outback region of north Queensland. Environ Toxicol 14:179–182.

Schembri MA, Neilan BA, Saint CP (2001) Identification of genes implicated in toxin production in the cyanobacterium *Cylindrospermopsis raciborskii.* Environ Toxicol 16:413–421.

Seifert M, McGregor G, Eaglesham G, Wickramasinghe W, Shaw G (2007) First evidence for the production of cylindrospermopsin and deoxy-cylindrospermopsin by the freshwater benthic cyanobacterium, *Lyngbya wollei.* Harmful Algae 6:73–80.

Shaw GR, Seawright AA, Moore MA, Lam PKS (2000) Cylindrospermopsin, a cyanobacterial alkaloid: evaluation of its toxicological activity. Ther Drug Monit 22:89-92.

Shen XY, Lam PKS, Shaw GR, Wickramasinghe W (2002) Genotoxicity investigation of a cyanobacterial toxin, cylindrospermopsin. Toxicon 40:1499-1501.

Senogles P, Shaw G, Smith M, Norris R, Chiswell R, Mueller J, Sadler R, Eaglesham G (2000) Degradation of the cyanobacterial toxin cylindrospermopsin, from *Cylindrospermopsis raciborskii*, by chlorination. Toxicon 38:1203–1213.

Spoof L, Berg KA, Rapala J, Lahti K, Lepisto L, Metcalf JS, Codd GA, Meriluoto J (2006) First observation of cylindrospermopsin in *Anabaena lapponica* isolated from the Boreal Environment (Finland). Environ Toxicol 21:552–560.

Steyn PS, (1998) The biosynthesis of mycotoxins, Rev Méd Vét 149:469–478.

Stoner RD, Adamsa WH, Slatkin DN, Siegelman HW (1989). The effects of single L-amino acid substitutions on the lethal potencies of the microcystins. Toxicon 27: 825-828.

Stirling DJ, Quilliam MA (2001) First report of the cyanobacterial toxin cylindrospermopsin in New Zealand. Toxicon 39:1219–1222.

Terao K, Ohmori S, Igarashi K, Ohtani I, Watanabe MF, Harada KI, Ito E, Watanabe M (1994) Electron microscopic studies on experimental poisoning in mice induced by cylindrospermopsin isolated from blue-green alga *Umezakia natans*. Toxicon 32:833-843.

Tillett D, Dittmann E, Erhard M, von Döhren H, Börner T, Neilan BA (2000) Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC 7806: An integrated peptidepolyketide synthetase system. Chem Biol 7:753–764.

Trogen GB, Annila A, Eriksson J, Kontteli M, Meriluoto J, Sethson I, Zdunek J, Edlung U (1996) Conformational studies of microcystin-LR using NMR spectroscopy and molecular dynamics calculations. Biochemistry 35: 3197-3205.

Ueno Y, Nagata S, Tsutsumi T, Hasegawa A, Watanabe MF, Park H-D, Chen G-C, Chen G, Yu S-Z (1996) Detection of microcystins, a bluegreen algal hepatotoxin, in drinking water sampled in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay. Carcinogenesis 17:1317-1321.

Van Egmond, H. P. (1983). Mycotoxins in dairy products. *Food Chemistry,* No. 11, pp. (289-307) *Pasteur Tunis,* No. 54, pp. (381-390).

Vasconceles V, Olivera S, Teles FO (2001) Impact of a toxic and non-toxic strain of Microcystis aeruginosa on the crayfish Procambarus clarkii. Toxicon 39:1461-1470.

Visconti A, Minervini F, Lucifero G, Gambatesa V (1991) Cytotoxic and immunotoxic effects of Fusarium mycotoxins using a rapid colorimetric bioassay. Mycopathologia 113:181–186.

Wang L1, Liu L, Zheng B (2013) Eutrophication development and its key regulating factors in a water-supply reservoir in North China. J Environ Sci (China) 25(5):962-70.

Welker M, Khan S, Haque MM, Islam S, Khan NH, Chorus I, Fastner J (2005) Microcystins (cyanobacterial toxins) in surface waters of rural Bangladesh: pilot study. J Water Health 3(4):325-37.

Welker, M, von Döhren, H (2006) Cyanobacterial peptides – nature's own combinatorial biosynthesis. FEMS Microbiol Rev 30:530–563.

Wiseman DW, Marth EH (1983) Heat and acid stability of aflatoxin M1 in naturally and artificially contaminated milk. Milchwissenschaft 38:464-466.

World Health Organization (1998). Guidelines for drinking water quality, 2 ed. Addendum to Vol. 1, pp. 13-14. Recommendations. Geneva, World Health Organization, 1998.

Zegura B, Sedmak B, Filipic M (2003) Microcystin-LR induces oxidative DNA damage in human hepatoma cell line HepG2. Toxicon 41:41-48.

Zimba PV, Khoo L, Gaunt PS, Brittain S, Carmichael WW (2001) Confirmation of catfish, *Iactalurtus punctatus* (Rafinesque) mortality from Microcystis toxins.. Journal of fish desease 24(1):41-47.

STATE OF ART

1.1 T-2 and HT-2 analysis methods-techniques

There is a need to develop sensitive and accurate analytical methods for measuring these mycotoxins in cereals and cereal-based products in order to properly assess the risk of human and animal exposure.
Currently analytical methods used for mycotoxin analysis include thin layer chromatography (TLC) (Betina 1993; Krska et al. 2001), enzyme-linked immunosorbent assay (ELISA) (Ware et al. 1999; Thirumala-Devi et al. 2001; Heber et al. 2001), gas chromatography (GC) with electron capture (Langseth & Rundberget 1998) or mass spectrometric (Schwadorf & Müller 1992; Langseth & Rundberget 1998; Valenta 1998; Shephard 1998; Tanaka et al. 2000; Nielsen & Thrane 2001; Soleas et al. 2001) detection, liquid chromatography with fluorescence detection (LC-FLD) (Valenta 1998; Shephard 1998; Krska & Josephs 2001), and liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Young & Lafontaine 1993; Thakur & Smith 1994; Biselli et al. 2005).
LC-MS/MS appears to be most promising as a highly specific, broadly applicable detection method that provides both qualitative and quantitative data. Considering the possible contamination of foodstuffs by several mycotoxin producing fungal species and the production of different types of mycotoxins by one mould, a trend is to develop methods suitable for the determination of several mycotoxins in a single run (Sewram et al. 1999; Monti et al. 2000; Rundberget & Wilkins 2002; Royer et al. 2004; Berthiller et al. 2005; Cavalière et al. 2005; Kokkonen et al. 2005; Sorensen & Elbaek 2005; Abbas et al. 2006; Delmulle et al. 2006; Sulyok et al. 2006; Spanjer et al. 2008). Currently published protocols, however, have been developed and optimized for different foods and feeds.
As to *Fusarium* trichothecenes, they are normally classified into two types: type-B trichothecenes all have a ketone functionality at the 8-carbon position, while the type-A trichothecenes have other types of functionalities (e.g., hydroxyl or ester) at the C-8 position. Gas-chromatographic (GC) and liquid-chromatographic (LC) methods coupled with mass spectrometry (MS), have been used for the analysis of type-A trichothecenes including T-2 toxin and HT-2 toxin (Pascale et al., 2007). High-performance liquid chromatography (HPLC) with UV detection is generally not applicable to sensitive detection of type-A trichothecenes due to the lack of a strong chromophore group within their structures (Jiménez et al. 2000). As T-2 toxin and HT-2 toxin also lack native fluorescence, HPLC methods have been developed using derivatizing reagents (for example, with coumarin-3-carbonyl chloride) and detection by fluorescence (Lippolis et al. 2007) or, more recently, by treating these analytes with cyclodextrins as modifiers of fluorescence (Maragos et al., 2008).
The need for derivatization is a disadvantage of some methods such as HPLC-FLD and gas chromatography. Cross-reactivity may occur in ELISA trials. In some cases, ELISA results are confirmed by HPLC-MS/MS, but in other cases there is the possibility of false positives. These methods are useful as screening and quantitative methods, but cannot be considered as confirmatory methods, For this reason, HPLC-MS/MS is the only technique to be used for confirmation purposes.
In more recent years, thanks to developments in mass spectrometry technology with the consequent improved selectivity and sensitivity, and the capacity to distinguish residues and contaminates from co-extractives, instrumental analysis now requires in some cases, minimal sample preparation using simple methods, with or without clean-up. The triple-quadrupole tandem MS (MS/MS) is considered the gold standard for quantitative analysis of multiple compounds. “Dilute-and-shoot” based approaches (Mol et al., 2008), based on modern liquid chromatography (LC) combined with MS/MS, became a popular analytical method with many applications, including mycotoxins analysis (Oueslati et al., 2012, Sulyok et al., 2010 and Zachariasova et al., 2010). The Dilute-and-shoot method has several practical benefits, such as minimizing analyte losses during purification, while increasing detection limits. In some situations, sample complexity can exceed the capability of MS/MS instrumentation and significantly impact the accuracy of a determination and ruggedness of method. This is especially true for mycotoxins, which are generally present at very low concentration. Therefore, methods with greater selectivity, such as high-resolution MS, accurate mass MS, or two-dimensional chromatography–MS, that can analyze samples with a minimum of pre-treatment, are necessary for the determination of these toxins.
As a general conclusion of the literature survey HPLC-ESI-MS/MS together with an efficient sample clean-up step seems to be the method of choice for mycotoxin analysis in food samples. The choice of HPLC-ESI-MS/MS with immunoaffinity clean-up can be explained in terms of method accuracy and sensitivity.
“Quick, Easy, Cheap, Effective, Rugged, and Safe” (QuEChERS) sample preparation approaches have been proposed for analysis of a wide range of matrices and analytes. Many procedures can provide high quality results and other benefits for the analysis of mycotoxins (Zachariasova et al., 2010).

1.2 Aflatoxin M_1 analysis methods-techniques

Currently most common analytical methods used for aflatoxin M_1 analysis are enzyme-linked immunosorbent assay (ELISA) (Thirumala-Devi et al., 2002, Vdovenko et al., 2014) for screening analysis purposes, and liquid chromatography with fluorescence detection (LC-FLD) (Santini et al., 2013; Tabari et al., 2013; Iha et al., 2011; Muscarella et al., 2011; Cattaneo et al., 2011) for confirmatory analysis purposes. The choice of HPLC-FLD detection with immunoaffinity clean-up is the best choice in terms of method accuracy and sensitivity. In order to

minimizing time of analysis so giving an analytical answer to the "Quick Response" need, we attempt to develop a new strategy based upon of HPLC-ESI-MS/MS with QuEChERS clean-up.

1.3 Microcystins and cylindrospermopsin detection and quantification

Several methods for the assessment of microcystin are available, enzyme–linked immunosorbent assays (ELISA) (An & Carmichael, 1994, Yu et al., 2002); inhibition of protein phosphatase (Dawson, 1998), direct DNA test to detect genetic sequences unique for the multi gene cluster required for toxin synthesis by polymerase chain reaction (PCR, Baker et al., 2002, Pan et al., 2002) and chemical assays, including HPLC, MS (Ferranti et al., 2009).
Also for cylindrospermopsin, both screening and confirmatory methods have been described in literature for analysing this cyanotoxin, based on enzyme-linked immunosorbent assay (ELISA) (Abraxis LLC, Warminster, PA, USA), molecular assays involving real-time PCR (Rasmussen et al., 2008), in vivo assays (essentially mouse assays)**,** cell line assays (Neumann et al. 2007, Froscio et al., 2009) and chemical assays, including HPLC-DAD (Harada et al. 1994; Hawkins et al. 1997) and HPLC-MS/MS techniques (Eaglesham et al., 1999, Saker et al., 2004, Gallo et al., 2009).

1.4 Microcystin chemical assays

Concentration/clean-up procedures for microcystins in water can be effected with C18 solid phase extraction cartridges and extracion/concentration/clean-up in more complex matrices can be performer with acidified methanol/water (Gallo et al., 2010). As the heterogeneity of the more than 90 microcystins, the hydrophobicity of the individual toxins can affect their recoveries. Recoveries can also be affected by the type of C18 extraction cartridge, and hence the cartridges to be used in an extraction/clean-up procedure must be evaluated with the toxins of interest.
Determination of the extracted microcystins is best carried out using HPLC procedures. HPLC effects separation of the individual toxins, the use of photo-diode array detection whereby the spectra of the eluted components are monitored assists in the identification of separated components as microcystins, but the possibility of coelution of different toxins is high. Co-extracted material may also coelute and therefore also interfere with the spectral analysis. Moreover, identification based on spectral characteristics may still be difficult at low toxin concentration.
Mass spectrometry as a detection technique following HPLC separation offers a better means of identifying separated components as microcystins, and in the case of MS/MS, it may be possible to better identify specific compounds. Increasing availability of suitable instrumentation and falling costs should see LC/MS being the method of choice for the routine determination of these toxins if standards are available.
There are a limited number of microcystin standards available commercially and these are not certified quantitative analytical standards. The availability of certified standards is currently a limitation for precise quantification of microcystins for all methods currently used, however this is likely to improve as commercial suppliers respond to the world wide demand for monitoring these toxic compounds in water supplies. The technique of MALDI fulfills other practical criteria both for use in routine monitoring of microcystin, having acceptable detection limits in relation to the guideline, good documentation with a degree of standardization and, in situations where standards are unavailable for particular toxins, or the microcystin is unknown, to identify the substance. The same is not true of MS/MS detection with mass analyzer such as ion trap, Q-Trap and Q-ToF, i.e., the total ion current depends markedly on the particular microcystin. Therefore concentrations of unknown microcystins, or microcystins for which standards are not available, cannot be estimated with this method.

1.5 Cylindrospermopsin chemical assays

Several chimical methods have been used so far to analyse CYN in cyanobacterial cultured species, in environmental samples of fresh and brackish water, including drinking water and recreational water bodies, and also in contaminated animal tissues.
The knowledge about the chemistry of CYN and on the problems associated with complex matrices of samples containing this toxin is crucial to select the most reliable identification, extraction, separation and analyses techniques to monitor this cyanotoxin in the environment.
Ohtani et al. (1992) reported bioassay-guided method to isolate CYN from cyanobacteria biomass by size-exclusion chromatography (Toyopearl HW40F) and identification/purification by HPLC-UV on a reverse-phase column at a distinct absorbance maximum of 262 nm, due to the uracil nucleus. Then, more detailed analytical and semi-preparative HPLC methods for the determination and purification of the toxin in the same sample type were reported (Harada et al. 1994; Hawkins et al. 1997). A much more sensitive and specific LC-MS/MS method was developed by Eaglesham et al. (1999) being able to determine trace amounts of CYN in water samples. Saker et al. were able to detect CYN in more complex matrix such as crayfish tissue (1999c) and mussel (2004) using the LC-MS/MS detection technique.

LC-MS/MS is the ideal method for small amounts of toxin and for complicated sample types, having, however, the disadvantage of being a costly approach. HPLC-PDA is one of the alternative methods to quantify the toxin and analogues present in cyanobacterial cultures and samples that do not produce any background like the case of the environmental samples (Welker et al. 2002). Another approach to obtain background free cell and animal tissue extracts is the addition of a clean-up step by SPE. Carmichael et al. (2001), did an extensive study to find the best SPE sorbent to retain CYN and deoxy-cylindrospermopsin to concentrate the toxin present in the media of *Cylindrospermopsin raciborskii* cultures. The graphitized carbon cartridges were most effective, being able to retain CYN very strongly and with the media concentration of 800 μg/L. Later, Kubo et al. (2005) successfully used a double system of cartridges, which consisted of a styrene polymer cartridge and an anion exchange cartridge. The cartridge double system was able to isolate both molecules from the same culture, CYN and deoxy-cylindrospermopsin. In our laboratories we were able to isolate CYN from water and fish muscle, the later extracted by a hexane liquid–liquid extraction followed by HLB-SPE, a hydrophilic–lipophilic balance reversed-phase sorbent. The extracts of both types of samples had excellent detection limits by LC/ESI-MS/MS, 0.04 ng/ml and 0.6 ng/g, respectively (Gallo et al., 2009).

1.6 Accumulation of microcystin and cylindrospermopsin in food

The bioaccumulation of toxins is the process that causes concentrations in tissue to be higher than in the environment, due to the uptake of the cyanobacterial toxins available through drinking, diet, and/or direct contact (all possible exposure routes). The biomagnification is the process by which toxin concentrations are increased through successive trophic level interactions (Kinnear, 2010). Moreover, bioconcentration means that concentrations are greater in the organism than in the water, where uptake can only be directly from the water (dissolved toxin) (Ibelings & Chorus, 2007). The potential bioaccumulation of cyanobacterial toxins in aquatic organisms and/or plants is of great concern, because these organisms will subsequently be consumed by humans, thus increasing the risk of intoxications.

Studies on the bioaccumulation of CYN and, particularly, MCC in animals and plants have increasingly been published in recent years. Most of them have been carried out on fish, crustaceans, bivalves, and snails. Nevertheless, more studies in both field and laboratory conditions have recently been made on bioaccumulation in plants. In many of these studies, however, the term bioaccumulation is used to refer to the presence of toxin in organisms. If more formal definitions of bioaccumulation, bioconcentration and biomagnification are used (Ibelings & Chorus, 2007), good evidence to support bioaccumulation of MCCs or CYN in aquatic food webs is scarce and inconclusive. Thus, in general, although seafood certainly contains cyanobacterial hepatotoxins and can pose a risk to the health of human consumers, this may be due to the uptake and accumulation in organs and tissue, and not to biomagnification. Biomagnification is a phenomenon that is commonly found with lipophilic toxicants, but it is less likely with hydrophilic compounds such as MCCs and CYN. Thus, Martins and Vasconcelos (2009) considered that the absence of clear magnification pattern of MCCs is due not only to their hydrophilic characteristics but also to the detoxification mechanisms present in most organisms, using the GST system to metabolize and excrete MCCs.

Several authors have discussed MCC accumulation in bivalves, crustaceans and gastropods in both laboratory and field conditions. Although most studies focus on determining MCC levels in the hepatopancreas (the main target for MCCs) and the whole body, some of them demonstrated MCC bioaccumulation in other organs. Kinnear (2010) reviewed all the published literature on the presence of CYN in animals and plants, including factors that may interfere in this process. For MCCs there is a considerable amount of information available on sources, bioaccumulation and effects of the toxins, but this is not the case for CYN.

For bivalves, some of the most remarkable field results were obtained in Lake Taihu (China), where four different species (*Anodonta woodiana, Hyriopsis cumingii, Cristaria plicata, Lamprotula leai*) were evaluated (Chen & Xie, 2005). These results showed that bioaccumulation patterns depend on the bivalve species because different levels of MCCs are found in each bivalve. The order of decreasing MCC contents (in μg/g dry weight, dw) for all these bivalves was hepatopancreas > intestine > gills > foot > rest. Recently, Vareli et al. (2012) published a report on the presence of MCCs in the Mediterranean Sea, and they observed that although MCC levels in water were low, the edible mussel *Mytilus galloprovincialis* accumulated high quantities of MCCs, exceeding the upper limit of the TDI of MCCs as determined by WHO, with the consequent risks to human.

1.7 Presence of cyanotoxins in cyanobacteria food supplements

Cyanobacteria are often linked to environmental disasters, and the poisoning of plants and animals (including human beings). However, they are also often used as food supplements. This is because cyanobacteria have a wide variety of nutrients that are beneficial to humans, such as amino acids, minerals, vitamins, fatty acids, β-carotenes and chlorophyll (Dittmann and Wiegand, 2006). Their wide variety of properties (anticancer, anti-inflammatory, hypolipidemic, antioxidant, antibacterial, antiviral activities, etc.) also make them useful for medical applications (Deng & Chow, 2010, Dittmann and Wiegand, 2006, Sharma et al., 2011). These properties mean that dietary supplements containing cyanobacteria are of great interest to society, so their safety in the long-term must be

properly evaluated. Furthermore, there is a potential risk of exposure to cyanotoxins through production cultures being contaminated with toxin-producing strains or mistaken for toxin-producing species (for example, MCCs) (Dittmann and Wiegand, 2006).
The most commonly used cyanobacteria in the production of food supplements are *Spirulina* sp. and non-toxic *Aphanizomenon flos-aquae*. Both types of cyanobacteria have been shown to be safe, but sometimes toxins can be generated by these species (Draisci et al., 2001) or by other contaminant cyanobacteria, such as *Microcystis aeruginosa* (Eisenbrand, 2008), which can cause health problems.

Spirulina sp.

Spirulina is the most commonly used cyanobacteria in food supplementation and the most frequently used species (also called Arthrospira) are *S. maxima*, *S. platensis* and *S. fusiformis* (Marles et al., 2011). *Spirulina* sp. has been commercialized as a human and animal dietary supplement, particularly since the 1990s and largely because of its fast growth, assimilability, and high concentration of nutrients (Jiang et al., 2008). It is mostly produced in China and India, where it is harvested from natural water bodies or cultivated in outdoor ponds (Rellán et al., 2009).
Because these cyanobacteria are harvested directly from natural environments, contamination may be caused by other toxic cyanobacteria such as Microcystis, Anabaena, Nostoc, Oscillatoria, and so forth (Jiang et al., 2008 and Rellán et al., 2009). In fact, it has been demonstrated that cyanobacterial food supplements may sometimes be mislabeled and contain other cyanobacterial genera not mentioned on the label (Saker et al., 2007). For this reason, commercial strains need to be analyzed for the presence of toxins, especially MCCs and anatoxin-a (a neurotoxin), in order to guarantee that they are safe for human consumption.
The Dietary Supplements Information Expert Committee (DSI-EC) of the United States Pharmacopeial Convention (USP) reviews the safety of dietary supplement ingredients for the purpose of determining whether they should be admitted as quality monographs into the United States Pharmacopeia and National Formulary (USP-NF). Considering the data reviewed and because of the limited information available in the adverse event reports, the DSI-EC unanimously voted for a class A safety assignment for *S. maxima* and *S. platensis*, which suggests that the available evidence does not indicate that they are a serious risk to health or other public health concern that precludes admission of quality monographs into USP-NF when these dietary ingredients are properly identified, formulated, and used (Marles et al., 2011).

Aphanizomenon flos-aquae

Another cyanobacteria species that is suitable for human consumption is *Aphanizomenon flos-aquae* (AFA). It is mainly produced in the USA in natural lakes, because it is difficult to grow under laboratory conditions (Carmichael et al., 2000 and Gilroy et al., 2000). As a result, it can be contaminated by other cyanobacteria species capable of producing cyanotoxins (*Microcystis aeruginosa*, *Aphanizomenon ovalisporum*), which is a risk to humans.
Several studies have been made to elucidate the risk of AFA consumption, (Gilroy et al., 2000, Lawrence et al., 2001, Preuβel et al., 2006, Saker et al., 2007, Vinogradova et al., 2011). The first report on the presence of cyanotoxins in AFA dates back to 1996, when MCCs from *M. aeruginosa* were found in AFA supplements (Gilroy et al., 2000). Subsequently, a safe MCC level of 1 μg/g in blue-green algae food supplements was established (Gilroy et al., 2000).

Figure 5. Klamath Lake (Oregon)

1.8 Cyanotoxins studies conducted so far in our laboratories – microcystins and cylindrospermopsin in italian lakes

As to microcystins, in our laboratories we firstly developed a method for the analysis of four microcystins by ion-trap LC-MS and MALDI-TOF/MS: MC-RR, MC-LR, MC-LW, and MC-LF. Method for MCC determination in water and cyanobacterial samples turned out simple, rapid, reliable, shown satisfactory mean recoveries and limit of quantification levels in water down to 0.5-2.0 ng/ml, with the performances of both MS detector comparable.
The method was applied to the analysis of water and algae samples from Lake Averno, near Naples, as a consequence of a cyanobacterial bloom of *Planktothrix rubescens*, and results demonstrates that ion trap detection only excludes the presence of the four microcystins, whereas MALDI-TOF/MS detected three main compounds. Moreover, on the basis of their fragmentation patterns, two substances were identified as anabaenopeptin B and anabaenopeptin F, which are less toxic than microcystins.
So the analysis of water and algae cell extracts showed no contamination by known microcystins in Lake Averno, but other substances were detected with MALDI-TOF/MS technique, which turned out as the most successful for screening of biotoxins in the samples. The method could characterize the biotoxins produced in Lake Averno for evaluation health risks related to their presence (Ferranti et al., 2009).
Further investigation were performed, in which method performances were evaluated and compared for the analysis of four microcystins in freshwaters with three MS-based methods: ion trap, MALDI-TOF, and quadrupole time-of-flight (Q-TOF). In particular, a liquid chromatography/electrospray ionisation (LC/ESI)-Q-TOF-MS/MS method was firstly described to analyze MCCs in freshwaters. Method performances were tested in terms of limit of detection, limit of quantification, mean recoveries, repeatability and specificity. Method comparison with the different mass analyzers proved LC/ESI-Q-TOF-MS/MS is the most sensitive and repeatable, allowing for highly selective analysis of MCs. The unknown compound produced by *Planktothrix rubescens* in Lake Averno was than identified on the basis of its ionization LC/ESI-Q-TOF-MS/MS spectrum. The study of the fragmentation pattern proved the compound was a microcystin never descrive before; its complete molecular structural formula was elucidated as [9-acetyl-Adda]-MC-RR, showing a measured molecular mass at 1065.58 Da. This result was confirmed by the peak at *m/z* 1066.5478 in the MALDI-TOF spectrum. The combination of MALDI-TOF-MS and LC/ESI-Q-TOF-MS/MS techniques proved to be an effective, sensitive and rapid analytical strategy for characterising the contamination of cyanotoxins. MALDI-TOF-MS analysis allows for rapid analytical strategy for characterising the contamination of cyanotoxins. MALDI-TOF-MS analysis allows for rapid screening over a wide molecular mass range, and preliminarly identification of cyanobacteria biotoxins; a more accurate analysis of the contamination profile attained by LC/ESI-Q-TOF-MS/MS, that proved to be a potent tool for characterisation of both known and unknown MCCs, living the opportunity to focus on each compound for further analytical study (Ferranti et al. 2009).
Later these rapid, straightforward and accurate analytical strategies were estended for the analysis of seven MCCs (MC-RR, MC-LR, MC-YR, desMe-MC-RR, desMe-MC-LR, MC-LW, and MC-LF) were successfully employed to study an important *Planktothrix rubescens* bloom in the dam of Lake Occhito (Southern Italy), that supplies drinking water and water for agricultural works in the area around the town of Foggia. MALDI-TOF-MS analysis of cell extracts allowed for identification of the main cyanotoxins produced. DesMe-MC-RR and MC-RR were recognised as the more aboundant microcystins; in particular, the first one was largely present in most samples and can therefore be considered as representative of this bloom. A complete "profiling" of the cyanotoxins produced was assessed, by identifying also the presence of several anabaenopeptins and aeruginosides; some novel compounds belonging to these groups were also identified. The same microcystin contamination profile was observed in all water sample collected. Accurate, reliable, quantitative analysis was performed by LC/ESI-Q-TOF-MS/MS, obtaining full information about the contamination levels in water samples from the dam over three weeks. LC/ESI-Q-TOF-MS/MS allowed confirmly unambigously the presence of the main MCCs detected, on the basis of their MS/MS spectra.
The study proved that the analytical strategy adopted allows for rapid identification of cyanotoxins present in a cell extract, even in the early step of contamination, and to focus attention for further monitoring of the most abundant and/or harmful compound released in the water, that could represent a risk for the consumers (Ferranti et al., 2013).
Further investigation of pollution was also developed on a few commercial fish species downstram of the lake were performed. For this purpose, an extraction and clean-up method of five microcystin (MC-RR, MC-LR, MC-YR, MC-LW, MC-LF) from fish muscle previousely developed in our laboratories was applicated to fish species collected in the area of Lake Occhito, but without estabilishing the presence of MCCs. Further investigation of pollution also in mussel cultivations downstram of the lake Occhito were performed. For this purpose, the extraction and clean-up method of microcystin from fish muscle was extended to two demethylated MCCs variants (desMe-MC-RR, desMe-MC-LR) and applicated to mussel collected in the area of Lake Occhito. It was revealed the presence of both two demethylated forms in several samples at concentration ranging between 5 and 12 ng/g, which were indicated by recent studies as eventually more toxic than their fully methylated counterparts (Ufelmann et al., 2012).

As to cylindrospermopsin, methods for unambiguous identification and quantitative analysis in freshwaters and fish muscle by liquid chromatography coupled to ion trap mass spectrometry (LC/ESI-MS/MS) were developed. Method performances in terms of the limits of detection, the limits of quantification, the specificity, mean recoveries, repeatability and within-laboratory reproducibility were evaluated. Results showed that the methods were simple, rapid, reliable and shown satisfactory mean recoveries and limit of quantification levels in water down 1.0 ng/ml, and down 2.0 ng/ml in fish muscle (Gallo et al., 2009).
The method was successfully applied for identification and quantification of cylindrospermopsin in extract from cyanobacteria cells collected in Lake Averno, near Naples, reported as the first case of this cyanotoxin in Southern Italy. Unfortunately, the species producing CYN was not identified, maybe because of a negligible abundance in respect to *Microcystis aeruginosa*; in the literature, it was reported that *Microcystis spp.* and *Aphanizomenon spp.* can occur contemporarily during the same bloom. Similarly, cylindrospermopsin has been detected also in middle Italy (Lake Albano) and Sicily (Lake Pergusa).
Determination of cylindrospermopsin in freshwaters have been developed also with MALDI-TOF-MS technique and LC/ESI-Q-TOF-MS/MS. All these MS detectors can confirm cylindrospermopsin down to sub-ppb levels, allowing for monitoring early contamination stages, at concentration quite below the guide value suggested for freshwaters. Also the methods with MALDI-TOF-MS and LC/ESI-Q-TOF-MS/MS techniques were successfully applied to detection and time monitoring of cylindrospermopsin in water samples and extracts from cyanobacteria in Lake Averno, Lake Albano and Lake Pergusa, with a good agreement in quantitative determination with all the three techniques.

1.9 Screening and confirmatory methods - criteria approach

The control of substances is regulamented by law. European regulations concern different aspects: legislative limits of drugs, toxins in different products, choice of sampling and analysis methods, requirements for the laboratories designed with the official control of food products and requirements for analytical methods performances.
As a result of advances in analytical chemistry, in the frame of Official Control, the concept of routine methods and reference methods has been superseded by criteria approach, in which performance criteria and procedures for the validation of screening and confirmatory methods are established.
Screening method means methods that are used to detect the presence of a substance or class of substances at the level of interest. These methods have the capability for a high sample throughput and are used to sift large numbers of samples for potential non-compliant results. They are specifically designed to avoid false compliant results.
Confirmatory method means methods that provide full or complementary information enabling the substance to be unequivocally identified and if necessary quantified at the level of interest.
Confirmatory methods for organic residues or contaminants shall provide information on the chemical structure of the analyte. Consequently methods based only on chromatographic analysis without the use of spectrometric detection are not suitable on their own for use as confirmatory methods. However, if a single technique lacks sufficient specificity, the desired specificity shall be achieved by analytical procedures consisting of suitable combinations of clean-up, chromatographic separation(s) and spectrometric detection.
As a result, the criteria approach allowed the continuous application of new methods, analytical techniques, and analytical equipment, thereby allowing tracking of scientific developments more easily.
Laboratories were able to apply their own methods and introduce new techniques. This also led to the introduction of in-house validation, i.e., the verification of the fitness for purpose of individual methods, which in turn requires the establishment of criteria for performance assessment.

1.10 Method validation

Within-laboratory method validation is a serie of experiments performed to provide evidence that a method is fit for the purpose for which it is to be used.
The process of characterising the performance to be expected of a method is intended in terms of its scope, specificity, accuracy (bias), sensitivity, repeatability and within laboratory reproducibility.
Method validation is a requirement of accreditation bodies, and must be supported and extended by method performance verification during routine analysis (analytical quality control and on-going method validation). All procedures (steps) that are undertaken in a method should be validated, if practicable.
The modern approach of a method validation also introduced several new aspects distinct from its predecessors, with particular regard to the identification of drugs, residues or contaminants of interest and a complete new approach to validation. In the old legislation, the criteria, which were assessed for identification purposes, were the retention time, relative retention time, and spectra resulting from the use of different analytical techniques (UV and MS). When selected ion monitoring (low resolution) was applied, at least four ions with a particular ratio had to be present to claim a result as “positive.” In reaction to this superseded legislation, the 2002/657/EC introduced a system of identification points and laid down permitted tolerances for the relative intensities of the detected ions. Moreover, the result expressions “positive” and “negative” were replaced by “compliant” and “noncompliant”

which fit better with the different substance groups under consideration in Council Directive 96/23/EC, Annex I. Indeed, substances with maximum residue limit (MRL) may be present (positive), but at concentrations below their respective MRL values, and thus “compliant.”

1.11 REFERENCES

Abbas HK, Cartwright RD, Xie W, Shier WT (2006) Aflatoxin and fumonisin contamination of corn (maize, *Zea mays*) hybrids in Arkansas. Crop Prot 25:1-9.

An J, Carmichael WW (1994). Use of a colorimetric protein phosphatase inhibition assay and enzyme linked immunosorbent assay for the study of microcystins and nodularins. Toxicon 32:1495-1507.

Baker LC (2002) Cyanobacterial harmful algal blooms (CyanoHABs): Developing a public health response. Lake and Reservoir Management 18:20-31.

Berthiller F, Schuhmacher R, Buttinger G, Krska R (2005) Rapid simultaneous determination of major type A- and B-trichothecenes as well as zearalenone in maize by high performance liquid chromatography–tandem mass spectrometry. J Chromatogr A 1062:209-216.

Betina V (1993) Chromatography of mycotoxins: Techniques and applications. J Chromatogr Libr 54:141-149.

Biselli S, Hartig L, Wegener H, Hummert C (2005) Analysis of fusarium toxins using LCMS-MS: Application to various food and feed matrices. LC GC North America 23:404-416.

Carmichael WW, Drapeau C, Anderson DM (2000). Harvesting of *Aphanizomenon flos-aquae* Ralfs ex Born. & Flah. Var. flos-aquae (cyanobacteria) from Klamath Lake for human dietary use. J Appl Phycol 12:585–595.

Carmichael WW, Azevedo SMFO, An JS, Molica RJR, Jochimsen EM, Lau S, Rinehart KL, Shaw GR (2001) Human fatalities from cyanobacteria, chemical and biological evidence for cyanotoxins. Environ Health Perspect 109:663–668.

Cattaneo TM, Marinoni L, Barzaghi S, Cremonesi K, Monti L (2011) Testing the suitability of different high-performance liquid chromatographic methods to determine aflatoxin M1 in a soft fresh Italian cheese. J Chromatogr A 1218: 4738-4745.

Cavalière C, D'Ascenzo G, Foglia P, Pastorini E, Samperi R, Lagana A (2005) Determination of type B trichothecenes and macrocyclic lactone mycotoxins in field contaminated maize. Food Chem 92:559-568.

Chen J, Xie P (2005) Seasonal dynamics of the hepatotoxic microcystins in various organs of four freshwater bivalves from the large eutrophic lake Taihu of subtropical China and the risk to human consumption. Environ Toxicol 20:572–584.

Commission Decision 2002/657/EC of 12 August 2002 (2002) Off J Eur Commun. 221:8–36.

Council Directive 96/23/EC (1996) concerning the performance of analytical methods and the interpretation of results. Off J Eur Commun 125:10–31.

Delmulle B, De Saeger S, Adams A, De Kimpe N, Van Peteghem C (2006) Rapid Commun Mass Spectrom 20:771-776.

Deng R, Chow TJ (2010) Hypolipidemic, antioxidant, and anti-inflammatory activities of microalgae Spirulina. Cardiovasc Ther 28:33–45.

Dittmann E, Wiegand C (2006) Cyanobacterial toxins – occurrence, biosynthesis and impact on human affairs. Mol Nutr Food Res 50:7–17.

Draisci R, Ferretti E, Palleschi L, Marchiafava C (2001) Identification of anatoxins in blue-green algae food supplements using liquid chromatography–tandem mass spectrometry. Food Addit Contam 18:525–531.

Eaglesham GK, Norris RL, Shaw GR, Smith MJ, Chiswell RK, Davis BC, Neville GR, Seawright AA (1999) Use of HPLC-MS/MS to monitor cylindrospermopsin, a blue-green algal toxin, for public health purposes. Environ Toxicol 14:151–154.

Eisenbrand G (2008) Microcystins in Algae Products used as Food Supplements. Senate Commission on Food Safety.

Ferranti P, Fabbrocino S, Cerulo MG, Bruno M, Serpe L, Gallo P (2008) Characterisation of biotoxins produced by a cyanobacteria bloom in Lake Averno using two LC-MS-based techniques. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 25: 1530-7.

Ferranti P, Fabbrocino S, Nasi A, Caira S, Bruno M, Serpe L, Gallo P (2009) Liquid chromatography coupled to quadrupole time-of-flight tandem mass spectrometry for microcystin analysis in freshwaters: method performances and characterisation of a novel vatiant of microcystin-RR. Rapid Commun Mass Spectrom 23(9):1328-1336.

Ferranti P, Fabbrocino S, Chiaravalle E, Bruno M, Basile A, Serpe L, Gallo P (2012) Profiling Microcystins contamination in a water reservoir by MALDI-ToF and liquid chromatography coupled to Q/TOF tandem mass spectrometry. Food Res. Int. 54: 1321-1330.

Froscio SM, Fanok S, Humpage AR (2009) Cytotoxicity screening for the cyanobacterial toxin cylindrospermopsin. J Toxicol Environ Health A 72:345–349.

Gallo P, Fabbrocino S, Cerulo MG, Ferranti P, Bruno M, Serpe L (2009) Determination of cylindrospermopsin in freshwaters and fish tissue by liquid chromatography coupled to electrospray ion trap mass spectrometry Rapid Commun Mass Spectrom. 23: 3279–3284.

Gilroy DJ, Kauffman KW, Hall RA, Huang X, Chu FS (2000) Assessing potential health risks from microcystin toxins in blue-green algae dietary supplements. Environ Health Perspect 108:435–439.

Harada KI, Ohtani I, Iwamoto K, Suzuki M, Watanabe MF, Watanabe M, Terao K (1994) Isolation of cylindrospermopsin from a cyanobacterium *Umezekia natans* and its screening method. Toxicon 32:73–84.

Hawkins PR, Chandrasena NR, Jones GJ, Humpage AR, Falconer IR (1997) Isolation and toxicity of *Cylindrospermopsis raciborskii* from an ornamental lake. Toxicon 35:341–346.

Heber D, Lembertas A, Lu QY, Bowerman S, Co VLW (2001) An analysis of nine proprietari Chinese red yeast rice dietary supplements: Implications of variability in chemical profile and contents. J Altern Complem Med 7:133-139.

Ibelings BW, Chorus I (2007) Accumulation of cyanobacterial toxins in freshwater "seafood" and its consequences for public health. Environ Pollut 150:177–192.

Iha MH, Barbosa CB, Favaro RM, Trucksess MW (2011) Chromatographic method for the determination of aflatoxin M1 in cheese, yogurt, and dairy beverages. J AOAC Int 94: 1513-1518.

Jiang Y, Xie P, Chen J, Liang G (2008) Detection of the hepatotoxic microcystins in 36 kinds of cyanobacteria Spirulina food products in China. Food Addit Contam 25:885–894.

Jiménez M, Mateo JJ, Mateo R (2000) Determination of type A trichothecenes by high-performance liquid chromatography with coumarin-3-carbonyl chloride derivatisation and fluorescence detection. J Chromatogr A 870:473–481.

Kinnear S (2010) Cylindrospermopsin: a decade of progress on bioaccumulation research. Mar. Drugs 8:542–564.

Kokkonen M, Jestoi M, Rizzo A (2005) Determination of selected mycotoxins in mould cheeses with liquid chromatography coupled to tandem with mass spectrometry. Food Addit Contam 22:449-456.

Krska R, Baumgartner S, Josephs R (2001) The state-of-the-art- in the analysis of type A- and B-trichothecene mycotoxins in cereals. Fresenius J Anal Chem 371:285-299.

Krska R, Josephs R (2001) The state-of-the-art in the analysis of estrogenic mycotoxins in cereals. Fresenius J Anal Chem 369:469-476.

Kubo T, Sano T, Hosoya K, Tanaka N, Kaya K (2005) A new simply and effective fractionation method for cylindrospermopsin analyses. Toxicon 46:104–107.

Langseth W, Rundberget T (1998) Mycotoxin production and cytotoxicity of Fusarium strains isolated from Norwegian cereals. J Chromatogr A 815:103-113.

Lippolis V, Pascale M, Maragos CM, Visconti A (2007) Improvement of detection sensitivity of T-2 and HT-2 toxins using different fluorescent labeling reagents by high-performance liquid chromatography. Talanta 74:1476–1483.

Martins JC, Vasconcelos VM (2009) Microcystin dynamics in aquatic organisms. J Toxicol Environ Health B 12:65–82.

Marles RJ, Barrett ML, Barnes J, Chavez ML, Gardiner P, Ko R, Mahady GB, Dog TL, Sarma ND, Giancaspro GI, Sharaf M, Griffiths J (2011) United States pharmacopeia safety evaluation of Spirulina. Crit Rev Food Sci Nutr 51:593–604.

Maragos CM, Appell M, Lippolis V, Visconti A, Catucci L, Pascale M (2008) Use of cyclodextrins as modifiers of fluorescence in the detection of mycotoxins. Food Addit Contam Part A 25:164-71.

Mol HGJ, Plaza-Bolanos P, Zomer P, de Rijk TC, Stolker AAM, Mulder PPJ (2008) Toward a generic extraction method for simultaneous determination of pesticides, mycotoxins, plant toxins, and veterinary drugs in feed and food matrixes Analytical Chemistry 80:9450–9459

Monti SM, Fogliano V, Logrieco A, Ferracane R, Ritieni A (2000) Simultaneous determination of beauvericin, enniatins, and fusaproliferin by high performance liquid chromatography. J Agric Food Chem 48:3317-3320.

Muscarella M, Magro SL, Palermo C, Centonze D (2011) A confirmatory method for aflatoxin M1 determination in milk based on immunoaffinity cleanup and high-performance liquid chromatography with fluorometric detection. Methods Mol Biol 739: 195-202.

Neumann C, Bain P, Shaw G (2007) Studies on the comparative in vitro toxicology of the cyanobacterial metabolite deoxycylindrospermopsin. J Toxicol Environ Health A 70:1679–1686.

Nielsen KF, Thrane U (2001) Fast methods for screening of trichothecenes in fungal cultures using gas chromatography–tandem mass spectrometry. J Chromatogr A 929:75-87.

Ohtani I, Moore RE, Runnegar MTC (1992) Cylindrospermopsin, a potent hepatotoxin from the bluegreen alga *Cylindrospermopsis raciborskii*. J Am Chem Soc 114:7942–7944.

Oueslati S, Romero-González R, Lasram S , Frenich AG, Vidal JLM (2012) Multi-mycotoxin determination in cereals and derived products marketed in Tunisia using ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry Food and Chemical Toxicology 50:2376–2381

Pan H, Song LR, Liu YD, Borner T (2002) Detection of hepatotoxic *Microcystis* strains by PCR with intact cells from both culture and environmental samples. Arch Microbiol 178:421-427.

Pascale M, Lippolis V, Maragos CM, Visconti A (2008) Recent developments in trichothecene analysis. in: acs symposium series 1001 - food contaminants: mycotoxins and food allergens. d.p. siantar, m.w. trucksess, p.m. scott, e.m. herman eds., american chemical society, washington, dc, 2008, pp. 192-210.

Preußel K, Stüken A, Wiedner C, Chorus I, Fastner J (2006) First report on cylindrospermopsin producing *Aphanizomenon flos-aquae* (Cyanobacteria) isolated from two German lakes. Toxicon 47:156–162

Rasmussen JP, Giglio S, Monis PT, Campbell RJ, Saint SP (2008) Development and field testing of a realtime PCR assay for cylindrospermopsin-producing cyanobacteria. J Appl Microbiol 104:1503–1515.

Rellán S, Osswald J, Saker M, Gago-Martinez A, Vasconcelos V (2009) First detection of anatoxin-a in human and animal dietary supplements containing cyanobacteria. Food Chem Toxicol 47:2189–2195.

Royer D, Humpf HU, Guy PA (2004) Quantitative analysis of *Fusarium* mycotoxins in maize using accelerated solvent extraction before liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry. Food Addit Contam 21:678-692.

Rundberget T, Wilkins AL (2002) Determination of *Penicillium* mycotoxins in foods and feeds using liquid chromatography–mass spectrometry. J Chromatogr A 964:189-197.

Santini A, Raiola A, Ferrantelli V, Giangrosso G, Macaluso A, Bognanno M, Galvano F, Ritieni A (2014) Aflatoxin M1 in raw, UHT milk and dairy products in Sicily (Italy). Food Addit Contam Part B Surveill. 6: 181-186.

Saker M, Eaglesham GK (1999c) The accumulation of cylindrospermopsin from the cyanobacterium *Cylindrospermopsis raciborskii* in tissues of the Redclaw crayfish *Cherax quadricarinatus.* Toxicon 37:1065-1077.

Saker M, Metcalf JS, Codd GA, Vasconcelos VM (2004) Accumulation and depuration of the cyanobacterial toxin cylindrospermopsin in the freshwater mussel Anodonta cygnea. Toxicon 43:185-194.

Saker ML, Welker M, Vasconcelos V (2007) Multiplex PCR for the detection of toxigenic cyanobacteria in dietary supplements produced for human consumption. Appl Microbiol Biotechnol 73:1136–1142

Schwadorf K, Müller HM (1992) Determination of α- and β-zearalenol and zearalenone in cereals by gas chromatography with ion-trap detection. J Chromatogr 595:259-267.

Sewram V, Nieuwoudt TW, Marasas WFO, Shephard GS, Ritieni A (1999) Determination of the *Fusarium* mycotoxins, fusaproliferin and beauvericin by high-performance liquid chromatography-electrospray ionization mass spectrometry. J Chromatogr A 858:175-185.

Sharma NK, Tiwari SP, Tripathi K, Rai AK (2011) Sustainability and cyanobacteria (blue-green algae): facts and challenges. J Appl Phycol 23:1059–1081.

Shephard GS (1998) Chromatographic determination of the fumonisin mycotoxins. J Chromatogr A 815:31-39.

Soleas GJ, Yan J, Goldberg DM (2001) Assay of Ochratoxin A in Wine and Beer by High-Pressure Liquid Chromatography Photodiode Array and Gas Chromatography Mass Selective Detection. J Agric Food Chem 49:2733-2740.

Sorensen LK, Elbaek TH (2005) Determination of mycotoxins in bovine milk by liquid chromatography tandem mass spectrometry. J Chromatogr B 820:183-196.

Spanjer MC, Rensen PM, Scholten JM (2008) LC-MS/MS multi-method for mycotoxins after single extraction, with validation data for peanut, pistachio, wheat, maize, cornflakes, raisins and figs. Food Addit Contam 25:472-489.

Sulyok M, Krska R, Schuhmacher R (2010) Application of an LC–MS/MS based multi-mycotoxin method for the semi-quantitative determination of mycotoxins occurring in different types of food infected by moulds. Food Chemistry 119:408–416

Tabari M, Tabari K, Tabari O (2013) Aflatoxin M1 determination in yoghurt produced in Guilan province of Iran using immunoaffinity column and high-performance liquid chromatography. Toxicol Ind Health 29: 72-76.

Tanaka T, Yoneda A, Inoue S, Sugiura Y, Ueno Y (2000) Simultaneous determination of trichothecene mycotoxins and zearalenone in cereals by gas chromatography–mass spectrometry. J Chromatogr A 882:23-28.

Thakur RA, Smith JS (1994) Analysis of fumonisin B1 by negative-ion thermospray mass spectrometry. Rapid Commun Mass Spectrom 8:82-88.

Thirumala-Devi K, Mayo MA, Reddy G, Tangni EK, Larondelle Y, Reddy DV (2001) Occurrence of ochratoxin A in black pepper, coriander, ginger and turmeric in India. Food Addit Contam 18:830-835.

Thirumala-Devi K, Mayo MA, Hall AJ, Craufurd PQ, Wheeler TR, Waliyar F, Subrahmanyam A, Reddy DV (2002) Development and application of an indirect competitive enzyme-linked immunoassay for aflatoxin M1 in milk and milk-based confectionery. J Agric Food Chem 50: 933-937.

Ufelmann H, Krüger T, Luckas B, Schrenk D (2012) Human and rat hepatocyte toxicity and protein phosphatase 1 and 2A inhibitory activity of naturally occurring desmethyl-microcystins and nodularins. Toxicology 293: 59-67.

Valenta H (1998) Chromatographic methods for the determination of ochratoxin A in animal and human tissues and fluids. J Chromatogr A 815:75-92.

Vasconceles V, Olivera S, Teles FO (2001) Impact of a toxic and non-toxic strain of Microcystis aeruginosa on the crayfish Procambarus clarkii. Toxicon 39:1461-1470.

Vareli K, Zarali E, Zacharioudakis GSA, Vagenas G, Varelis V, Pilidis G, Briasoulis E, Sainis I (2012) Microcystin producing cyanobacterial communities in Amvrakikos Gulf (Mediterranean Sea, NW Greece) and toxin accumulation in mussels (Mytilus galloprovincialis). Harmful Algae 15:109–118.

Vinogradova T, Danaher M, Baxter A, Moloney M, Victory D, Haughey SA (2011) Rapid surface plasmon resonance immunobiosensor assay for microcystin toxins in blue-green algae food supplements. Talanta 84:638–643

Vdovenko MM, Lu CC, Yu FY, Sakharov IY (2014) Development of ultrasensitive direct chemiluminescent enzyme immunoassay for determination of aflatoxin M1 in milk. Food Chem 158: 310-314.

Ware GM, Zhao Y, Kuan SS, Carman AS (1999) Preparative method for isolating alphazearalenol and Zearalenone using extracting disk. J AOAC Int. 82:90-94.

Welker M, Bickel H, Fastner J (2002) HPLC-PDA detection of cylindrospermopsin-opportunities and limits. Water Res 36:4659–4663.

Young JC, Lafontaine P (1993) Detection and characterization of fumonisin mycotoxins astheir methyl esters by liquid chromatography/particle-beam mass spectrometry. Rapid Commun Mass Spectrom 7:352-359.

Yu FY, Liu BH, Chou HN, Chu FS (2002) Development of a sensitive ELISA for the determination of microcystins in algae. J Agri Food Chem 50:4176-4182.

Zachariasova M, Lacina O, Malachova A, Kostelanska M, Poustka J, Godula M et al (2010) Novel approaches in analysis of Fusarium mycotoxins in cereals employing ultra performance liquid chromatography coupled with high resolution mass spectrometry. Analytica Chimica Acta 662:51–61.

2 Aims of the study

The ubiquitous nature of food contaminants requires ever-evolving, rapid screening and confirmatory methods that are robust in the face of complex matrices. Because of the excellent sensitivity and high selectivity of MS detection, LC–MS has attracted increasing attention as a technique for analyte detection in complex biological, environmental and food matrices, especially in view of recent national and international laws and regulations.
The mass spectrometric response in complex samples is often directly related to the influence of co-eluting compounds on the ionization efficiency of analytes. This can lead to decreased sensitivity, selectivity and accuracy of liquid chromatography–mass spectrometry (LC–MS/MS) analyses, especially when matrix composition varies from sample to sample. By applying to certain sample clean-up and/or chromatographic separation strategies LC–MS/MS method performance can be improved. In line with these approaches, this work will discuss some effective strategies to improve LC–MS/MS method performance, sometimes very simple, sometimes more complex. The usefulness of these approaches will be exemplified with our recent experiments in the analysis of mycotoxins and cyanotoxins in food matrices, feedingstuffs and dietary supplements.

2.1 Advances in mycotoxin analysis with QuEChERS extraction methods

Mycotoxins, the secondary metabolites of many fungi, can contamine the feed supply, with the possible results including husbandry losses and contaminated food products reaching the consumer. For the analysis of mycotoxins, dairy products present a particularly challenging matrix too, that requires significant extraction and clean-up to remove proteins and lipids.
The traditional options for these matrix can produce tedious workflows; we demonstrated that QuEChERS extraction can be successfully applied to food and feed samples containing mycotoxins, leading to consider this approach a potential remedy for the inherent drawbacks of traditional extraction if applied with powerful strumental detection techniques such as modern mass spectrometry.

2.2 Cyanotoxins analysis in cyanobacteria dietary supplements

Cyanobacteria are the oldest life forms on earth known to produce a broad spectrum of secondary metabolites. The functions/advantages of most of these secondary metabolites (peptides and alkaloids) are unknown, however, some of them have adverse effects in humans and wildlife, especially when ingested, inhaled or upon dermal exposure. Surprisingly, some of these cyanobacteria are ingested voluntarily. Indeed, for centuries mankind has used cyanobacteria as a protein source, primarily *Spirulina* species. However, recently also *Aphanizomenon flos-aquae* are used for the production dietary supplements, supposedly efficacious for treatment of various diseases and afflictions. Unfortunately, traces of neurotoxins and protein phosphatases (inhibiting compounds) have been detected in cyanobacterial dietary supplements, making these health supplements a good example for human exposure to a mixture of cyanobacterial toxins in a complex matrix. To do an evaluation of sanitary risk for human health due to exposure deriving from consumption of contaminated cyanobacteria and algae supplements, analytical strategy were developed and successfully applied to real samples.

3 Results and discussion

In this work, we developed an effective method for the analysis of T-2 and HT-2 mycotoxins in feedingstuffs by adapting the QuEChERS extraction and clean-up method coupled with HPLC–MS/MS with ESI-Ion Trap detection technique. We strived to optimize sample preparation conditions to accommodate the necessary performance criteria for the application. Method developed was eventually validated in compliance with the legal performance criteria and applied to the analysis of real samples of feedingstuffs and non-processed cereals supposed for animal feeding.
With the QuEChERS extraction and clean-up method coupled with HPLC–MS/MS with ESI-Ion Trap detection technique we also examined aflatoxin M_1 in milk derivative and compared our proposed method with current IAC methods to identify where replacements might be made.
As to cyanotoxins, we developed and in-house validated a method for the determination of seven microcystins in dietary supplements with HPLC–MS/MS with ESI-Ion Trap detection technique. In particular, we focused our attention on demethylated forms, which, to our best knowledge are more common in Europe and eventually more toxic then their fully methylated counterparts.
An analytical method for the determination of cylindrospermopsin in dietary supplements was also developed with ESI-Ion Trap detection technique.
We analyzed eleven products of different brands available on the Italian market in 2014 consisting of *Aph. flos-aquae*, *Spirulina* and *Chlorella* for the presence of MCCs and CYN using our developed and, eventually validated methods to assess the toxic hazard related to exposure to cyanotoxins by assumption of dietary supplements.

3.1 Topic 1 – Development, validation of confirmatory methods for the analysis of mycotoxins in feedingstuffs and analysis of real samples

The aim of the study was to develop and validate a new method for the determination of T-2 and HT-2 toxins in feedingstuffs with a mass spectrometry approach. In particular, we wanted to reduce the clean-up steps, which tipically use the immunoaffinity chromatography. To do this we have optimized an extraction step with acidified acetonitrile/water assisted by a salt-out treatment followed by a clean-up step in dispersive SPE with QuEChERS technique. In order to validate the method, blank and artificially spiked samples were extracted and analyzed in LC-ESI-ion trap technique with reverse phase chromatographic separation and mass spectrometric detection. Series of experiments allowed us to evaluate method performances in order to validate a confirmatory method for the determination of mycotoxins in feedingstuffs. Validate method was then used to analyze real sample in a monitory programme.

3.2 Matherials and methods 1

3.2.1 Reagents

Mycotoxins T-2 and HT-2 stock standard solutions at 100 µg/mL in acetonitrile were purchased from Biopure (biopure, Tulln,Austria). Water for the HPLC mobile phase was produced in a Milli-Q system (Millipore, Bedford, MA, USA). Acetic acid (98% grade), formic acid and ammonium acetate of analytical grade purity were from Carlo Erba (Carlo Erba, Milan, Italy). HPLC-grade acetonitrile and HPLC-grade methanol were from Merck (Darmstadt, Germany).
For extraction and partition a mixture of anhydrous $MgSO_4$, sodium citrate dibasic sesquihydrate (di-Na) and sodium citrate tribasic dehydrate (tri-Na), NaCl were purchased from Agilent (Agilent) in a balanced extraction kit. For clean-up using d-SPE, PSA and octadecyl (C18) sorbents were also obtained from Agilent (Agilent) in clean-up kit. Mixtures of salts and mixtures of d-SPE sorbents were purchased pre-weighed and stored in Teflon centrifuge tubes. 0.25 µm nylon syringe filter were from Chrom Tech (Chrom Tech Inc., Apple Valley, MN, USA).

3.2.2 Samples

Samples of different types of oat, barley, maize, rye were collected from local feed stores and milled to a flour consistency; compound feed were available in our laboratories because sent us for the Official Control for analysis of other contaminants or drugs, they were milled to a flour consistency and stored before analysis.

3.2.3 HPLC-MS/MS analysis - apparatus

Analysis were performed using a LC/ESI-MS/MS system equipped with a Surveyor LC Pump Plus (Quaternary Pump), a Surveyor Autosampler Plus and a LCQ Advantage ion trap mass spectrometer with an ESI source (ThermoFisher Scientific).
Chromatographic separation was performed by injecting 50 µL of samples onto a 150 × 4.60 mm 5 µm particle C18 Gemini stainless steel column (Phenomenex, Torrance, CA, USA), at 0.35 mL/min, using 0.1% acetic acid in H_2O MilliQ 5 mM ammonium acetate as mobile phase A and 0.1% acetic acid 5 mM ammonium acetate in methanol as mobile phase B. The elution was performed at a flow rate of 0.35 mL/min in gradient mode according the following programme: at time 0 50% mobile phase B, hold for 2 minutes, from 50% to 10% in 5 minutes, hold for 5 minutes, then back to 50% in 3 minutes and re-equilibrate in 5 minutes.
The mass spectrometer was periodically calibrated with solutions of ultramark, caffeine and Met-Arg-Phe-Ala peptide provided by the manufacturer. During the LC/ESI-MS/MS experiments, mass spectra were acquired in the positive ion mode. The instrument was daily calibrated for the analysis by tuning on HT-2 pseudo-molecular ion $[M+Na]^+$ m/z 447.1.
The LC/ESI-MS/MS analysis was performed by monitoring the signals of the precursor ion → product ion transitions (selected reaction monitoring, SRM mode) from the LC/ESI-MS/MS dataset. The diagnostic ions for MS/MS qualitative analysis are summarized in the following table:

Table 4. ESI Ion Trap ions for T2 and HT2 toxins identification

	T-2	HT-2
Precursor ion $[M+Na]^+$	489.1	447.1
	387.0	345.0
	327.0	286.0
	245.0	
Precursor ion $[M+NH_4]^+$	484.0	
	466.1	
	304.9	
	227.2	

The SRM transition selected for quantitative analysis were m/z 489.1 → 387.0 with a collision energy of 40% for T-2 toxin, and m/z 447.1 → 345.0 with a collision energy of 40% for HT-2 toxin.
The data were acquired and processed using the Xcalibur™ software, version 1.3, from ThermoElectron.

3.2.4 Standard solution preparation

A working standard mix of T-2 and HT-2 toxins was prepared at concentrations of 10 µg/mL in methanol and stored in amber-glass vials at 4 °C. This working standard solution was appropriately diluted to provide 7-point calibration standards in mobile phase A–B (1:1, v/v) at 5.0, 10.0, 25.0, 50.0, 100.0, 250.0 and 500.0 ng/mL. The calibration standards at the same concentrations were prepared using a blank sample as solvent to evaluate matrix effects. For the recovery and precision experiments, working standard mix at 10 µg/mL served as high and middle level spiking solution. Low level spiking solutions were prepared by dilution of the high spiking solution in methanol at 100 ng/mL.

3.2.5 Sample preparation

10 ± 0.05 g of finely milled samples were weighed into a 50 mL Teflon centrifuge tube, unprocessed rice samples were used for the reagent blanks and for spiked samples. 10 mL MilliQ water and 10 mL 10% acetic acid in ACN were added then the tube vortexed for 30 s. The tubes were shaked with an automatic horizontal shaker at maximum speed for 1 h to fully disperse the sample, then a mixture of pre-weighted anhydrous $MgSO_4$, NaCl and citrate buffer were added and the tube were shaked immediately and vigorously by hand for 1 min. Tubes were centrifuged at 3500 rpm (2180 rcf) for 5 min; for d-SPE clean-up, 8 mL of the ACN extract (upper layer) were transferred into a 15 mL centrifuge tube containing C18 (0.25 g) and PSA (0.4 g) sorbents, then the tubes were shaked vigorously by hand for 1 min and centrifuged at 3500 rpm for 5 min. 2 mL of the extract were transferred to a glass tube and evaporated to dryness at 40°C under a stream of N_2. 1 mL of mobile phase was added A/B (1:1, v/v) to reconstitute the extract, and then vortexed for 1 min. The extract were filtered using 0.25 µm nylon syringe filter into autosampler vials.

3.2.6 Method validation

The European Commission (EC, 2002 and EC, 2006) were used as for guidelines and criteria to assess the method validation. Selectivity was determined from t_R, ion ratios, and identification-points (IP) for each analyte.
Calibration standards were prepared by combining standard solutions into the neat solvent and blank matrix extracts (matrix-matching) to yield the desired concentrations in the range of 5–500 ng/mL for both analytes. Matrix effect of each analyte was estimated by calculating the difference of the linear best-fit slope, obtained from the matrix-matched calibration curve and solvent-based standards calibration curve, divided by the slope of the solvent-based standards calibration curve.
Recovery and precision studies were conducted by spiking blank samples with 6 × 2 replicates at each spiking level: 5 (low), 250 (middle), and 2000 µg/kg (high), on three separate days.
Recoveries were calculated from matrix-matched standard calibrations.
Firstly, we tested different QuEChERS techniques for detection T-2 and HT-2 mycotoxins, then recovery experiments were carried out. Blank and spiked samples were extracted and then analyzed using the same HPLC–MS/MS conditions as described above. Recoveries were calculated by comparison with matrix-matched standard calibrations.

3.2.7 Performance criteria, other requirements and procedures for analytical methods

3.2.7.1 The validation procedure: outline and example approaches

Validation is undertaken following the completion of the method development or before a method that has not been previously used is to be introduced for routine analysis. We distinguish between initial validation of a quantitative analysis method to be applied in the laboratory for the first time and to extension of the scope of an existing validated method for new analytes and matrices.

3.2.7.2 Initial full validation for a quantitative analysis method

Validation needs to be performed for all analytes within the scope of the method, for defined matrices (as far as they are within the claimed scope of the method and, subsequently applicable to sample analyzed in the laboratory) and for a concentrations range.
A typical example of the experimental set up of a validation is:
Sample set (sub samples from 1 homogenized sample)

- Reagent blank
- 1 unspiked sample
- 6 spiked samples at LOQ
- 6 spiked samples at the highest level of the range of applicability
- 6 spiked samples at an intermediate level

This experimental set must be repeted twice
Instrumental sequence:

- Calibration standards in solvent at LOQ level
- Calibration standards in matrix at LOQ level
- Reagent blank

For data evaluation calibrate and inject the sequence and quantify with calibration standards in solvent or in matrix.
From the data determine at least the parameters from Table 5, 6 and 7 and verify them against the criteria.

3.2.7.3 Identification

Selective detectors employed with GC or LC such as ECD, FPD, NPD, DAD and fluorescence, offer only limited specificity. Their use, even in combination with different polarity columns, does not provide unambiguous identification. These limitations may be acceptable for frequently found residues, especially if some results are also confirmed using a more specific detection technique. Such limitations in the degree of identification should be acknowledged when reporting the results.

Mass spectrometry coupled to chromatography

Mass spectrometry in conjunction with chromatographic separation is a very powerful combination for identification of an analyte in the extract. It simultaneously provides: retention time, ion mass/charge ratio and abundance data.

Requirements for chromatography

For GC-MS procedures, the chromatographic separation should be carried out using capillary columns. For LC-MS procedures, the chromatographic separation can be performed using any suitable LC column.
In either case, the minimum acceptable retention time for the analyte(s) under examination should be at least twice the retention time corresponding to the void volume of the column. The retention time (or relative retention time) of the analyte in the sample extract must match that of the calibration standard (may need to be matrix matched) within a specified window after taking into consideration the resolving power of the chromatographic system. The ratio of the chromatographic retention time of the analyte to that of a suitable internal standard, i.e. the relative retention time of the analyte, should correspond to that of the calibration solution with a tolerance of ± 0.5% for GC and ± 2.5% for LC.

Requirements for mass spectrometry (MS)

Reference spectra for the analyte should be generated using the instruments and techniques employed for analysis of the samples. If major differences are evident between a published spectrum and that generated within the laboratory, the latter must be shown to be valid. To avoid distortion of ion ratios, the response of the analyte ions must not overload the detector. The reference spectrum in the instrument software can originate from a previous injection

without matrix present, but preferably from the same batch. Identification relies on proper selection of diagnostic ions. The (pseudo) molecular ion is a diagnostic ion that should be included in the measurement and identification procedure whenever possible. In general, and especially in single MS, high m/z ions are more diagnostic than low m/z ions (e.g. m/z < 100).
However, high m/z ions arising from loss of water or from common moieties may be of little use. Although characteristic isotopic ions, especially Cl or Br clusters, may be of particular utility, the selected diagnostic ions should not exclusively originate from the same part of the parent molecule. Choice of diagnostic ions may change depending on background interferences.
Diagnostic ion chromatograms should have peaks (exceeding S/N 3:1) of similar retention time, peak shape and response ratio to those obtained from a calibration standard analysed at comparable concentration in the same batch. Chromatographic peaks from different diagnostic ions for the same analyte must overlap with each other. Where an ion chromatogram shows evidence of significant chromatographic interference, it must not be relied upon to quantify or identify residues. The ion that shows the best signal-to-noise ratio and no evidence of significant chromatographic interference should be used for quantification.
In case of full scan measurement, careful subtraction of background spectra, either manual or automatically by deconvolution or other algorithms may be required to ensure that the resultant spectrum of the chromatographic peak is representative. Whenever background correction is applied, this must be applied uniformly throughout the batch and should be clearly indicated.
Different types and modes of mass spectrometric detectors provide different degrees of selectivity, which relates to the confidence in identification.
The requirements for identification are given in the following tables. They should be regarded as guidance criteria for identification, not as absolute criteria to prove presence or absence of a compound.

Table 5. Identification requirements for different types of mass spectrometers

MS mode	Single MS (standard mass resolution)	Single MS (high resolution/high mass accuracy)	MS/MS
Typical systems (examples)	quadrupole, ion trap, time-of-flight (TOF)	TOF, Orbitrap, FTMS, magnetic sector	Triple quadrupole ion trap, hybride MS (e.g. Q-TOF, Q-trap)
Acquisition:	Full scan, Limited m/z range, Selected ion monitoring (SIM)	Full scan, Limited m/z range, Selected ion monitoring (SIM)	Selected/multiple reaction monitoring (SRM/MRM), full scan product-ion spectra
Requirements for identification:	≥ 3 diagnostic ions, (preferably including quasi molecular ion	≥ 2 diagnostic ions (preferably including the quasi molecular ion). Mass accuracy < 5 ppm. At least one fragment ion.	≥ 2 product ions

Table 6. Default recommended maximum permitted tolerances for relative ion intensities using a range of spectrometric techniques.

Relative intensity (% of the base peak)	LC-MSn (relative)
> 50%	± 20%
> 20% to 50%	± 25%
> 10% to 20%	± 30%
≤ 10%	± 50%

Larger tolerances may lead to a larger percentage of false positive results.

3.2.7.4 Requirements for quantitative analysis of mycotoxins in feedingstuffs

Where no specific methods for the determination of mycotoxin levels in foodstuffs are required by Community legislation, laboratories may select any method provided the selected method meets the tabled criteria for the quantitative determination (Commission Regulation 401-2006).

Table 7. Default recommended tolerances for repeatability,reproducibility and accuracy

Level µg/kg	T-2 toxin		
	RSDr %	RSDR %	Recovery %
50-250	≤ 40	≤ 60	60 to 130
> 250	≤ 30	≤ 50	60 to 130
Level µg/kg	HT-2 toxin		
	RSDr %	RSDR %	Recovery %
100-200	≤ 40	≤ 60	60 to 130
> 200	≤ 30	≤ 50	60 to 130

Validate method performances in terms of accuracy and precision (repeatability and reproducibility) should comply with these tabeled values.

3.2.7.5 Method validation - Robustness trial

Although the major factors contributing to variability of a method may be explored by the classical, one variable at a time procedure, examining the effect of less important factors can be accomplished by a simpler Youden Ruggedness Trial (Youden & Steiner, 1975) Robustness is the capacity of a method to remain unaffected by small deliberate variations in method parameters. The robustness of a method is evaluated by varying method parameters.
To evaluate and quantify the effect of small variation, 7 parameters that may affect the outcome of the analysis have to be selected and assigned reasonable modified values to them.
Then 8 runs (a single analysis that reflects a specified set of factor levels) have to be conducted utilizing the specific combinations of high and low values for the factors as follows, and record the result obtained for each combination. (It is essential that the factors be combined exactly as specified or erroneous conclusions will be drawn.)

Table 8. Run factor combinations measurement

Runs	Factors combination							
Run 1	A	A	A	A	a	a	a	a
Run 2	B	B	b	b	B	B	b	b
Run 3	C	c	C	c	C	c	C	c
Run 4	D	D	d	d	d	d	D	D
Run 5	E	e	E	e	e	E	e	E
Run 6	F	f	f	F	F	f	f	F
Run 7	G	g	g	G	g	G	G	g
Result Obtained	S	T	U	V	W	X	Y	Z

With the capital letter is indicated the factor value as reported in the method, with the lowercase letter is indicated the modified value of the factor.

To obtain the effect of each of the factors, the differences of the measurements containing the subgroups of the capital letters and the small letters have to be set up like this:

$$D_A = |A - a| = \frac{(S + T + U + V)}{4} - \frac{(W + X + Y + Z)}{4}$$

$$D_B = |B - b| = \frac{(S + T + W + X)}{4} - \frac{(U + V + Y + Z)}{4}$$

$$D_C = |C - c| = \frac{(S + U + W + Y)}{4} - \frac{(T + V + X + Z)}{4}$$

$$D_D = |D - d| = \frac{(S + T + Y + Z)}{4} - \frac{(U + V + W + X)}{4}$$

$$D_E = |E - e| = \frac{(S + U + X + Z)}{4} - \frac{(T + V + W + Y)}{4}$$

$$D_F = |F - f| = \frac{(S + V + W + Z)}{4} - \frac{(T + U + X + Y)}{4}$$

$$D_G = |G - g| = \frac{(S + V + X + Y)}{4} - \frac{(T + U + W + Z)}{4}$$

After an evaluation of the differences so obtained, it is possible to individuate critical factors, then a standard deviation of the differences D_i (S_{Di}) is calculated with the formula:

$$S_{Di} = \sqrt{2 \cdot \sum \left(\frac{Di^2}{7} \right)}$$

This value have to be compared with the reproducibility standard deviation S_r (inter die) at the same spiking level with a F test. If S_{Di} is significantly higher then S_r ($F_{obs} > F_{tab}$) the conclusion is that at least one of the factor influences the result.

It is possible to exactly individuate which factor is critical with a t-test:

$$t = \frac{\sqrt{n} \cdot |D_i|}{\sqrt{2} \cdot Sr}$$

where:

D_i = any standard deviation of the differences
n = number of experiment run for every factor for every level
S_r = reproducibility standard deviation S_r

If the t value observed is higher then the critical value of t tabled for a two-tailed test for number of degrees of freedom with which S_r was obtained (at the 95% level of confidence), it means that the tested factor is critical and it has to be reported in the method.

3.3 Results and discussion 1

3.3.1 Linearity and matrix effects

The linearity of response vs. concentration was evaluated using 7 matrix-matched calibration points (not including zero concentration) over the range of 5–500 ng/mL. The analytes gave average linear regression (R^2) values of >0.95 over the range of interest (Table 9).
Matrix effects were unavoidable and we were not able to eliminate them in the analysis. Table 9 shows our estimated matrix effects, calculated from the differences between the matrix-matched standards and solvent-based standards calibrations. T-2 and HT-2 toxins, that were ionized in positive mode as sodium or ammonium adducts showed similar trends of signal suppression (up to 50%). Moreover, matrix effects seemed to be independent on concentration. All these results indicated the necessity of using matrix-matched standards to quantify analytes, in order to compensate for matrix effects.

Table 9. Retention times (t_R) with a tolerance range, linear regressions (R^2) in matrix, matrix effects, and analytical limits for the T-2 and HT-2 toxins analyzed with the positive ion mode.

Analyte	Ionization mode	$t_R \pm$ tolerance value (min)	linear regressions (R^2) in matrix	matrix effects (%)	analytical limits (µg/Kg)
T-2-Na^+	positive	11.92 ± 0.30	0.971	-52	5
HT-2-Na^+	positive	10.94 ± 0.27	0.998	-54	5

3.3.2 Developing ESI-ion trap detection method of T-2 and HT-2 toxins

Our first goal was to develop a method of analysis of T-2 and HT-2 toxins in feedingstuffs and non-processed cereals supposed for animal feeding using up-to-date instruments. We started with optimizing MS/MS conditions with ESI Ion Trap mass spectrometer. We began to study the molecules by injecting a 1000 ng/mL dilution in H_2O/MeOH v/v 0.1% acetic acid, 5 mM ammonium acetate.
In the positive ion detection mode, the base peak of HT-2 toxin $[M+Na]^+$, with a m/z ratio of 447.10, in the full scan mass spectra served as the precursor ion for optimizing other source parameters, such as the capillary temperature and the capillary voltage, that were finally selected as 300°C and of 4.5 kV respectively.
By tuning on the peak of m/z 447.10, the instrument software automatically set the ion optics and tube lens potentials.
Then, working in the SRM (single reaction monitoring) ion mode, with the precursor ion m/z 447.10 for HT-2 toxin and 489.13 for T-2 toxin, we individuate a fragmentation pattern by optimizing the collision energy at a 40% (instrumental parameter) for T-2 toxin and 40% for HT-2 toxin, these served both for quantitative than for qualitative identification; also, additional diagnostic ion for T-2 toxin were obtained by T-2-NH_4^+ adduct fragmentation at collision energy of 35%.
The most intense fragment ion generated from the precursor ion was chosen as the quantifier ion (m/z 345.0 for HT-2 and 387.0 for T-2), whereas the other most intense ions were used as qualifier ion for identification (table 4).

3.3.3 Developing a QuEChERS extraction method of T-2 and HT-2 toxins from feedstuffing

Four different combination of extraction and dispersive SPE clean-up were tested to optimize the analysis of T-2 and HT-2 toxins from feedstuffing. To do this, we did an extraction of artificially spiked samples in duplicate at level of 250 ng/g with every method variant. Results revealed that the best results we obtained with method 3 in terms of accuracy. Another information we extract from this experiment was that in matrix standardization is foundamental.

Sample preparation-QuEChERS method 1

10 ± 0.05 g of finely milled samples were weighed into a 50 mL Teflon centrifuge tube, unprocessed rice samples were used for the reagent blanks and for spiked samples. 10 mL MilliQ water and 10 mL 10% formic acid in ACN were added then the tube vortexed for 30 s. The tube was shaked with an automatic horizontal shaker at maximum speed for 1 h to fully disperse the sample, then a mixture of pre-weighted anhydrous $MgSO_4$, NaCl and citrate buffer was added and the tube was shaked immediately and vigorously by hand for 1 min. The tube was centrifuged at 3500 rpm (2180 rcf) for 5 min; for d-SPE clean-up, 8 mL of the ACN extract (upper layer) were transferred into a 15 mL centrifuge tube containing C18 (0.25 g) and PSA (0.4 g) sorbents, then the tube was shaked vigorously by hand for 1 min and centrifuged at 3500 rpm for 5 min. 1 mL of the extract was transferred to a glass tube and evaporated to dryness at 40°C under a stream of N_2. 0.5 mL of mobile phase were added A/B (1:1, v/v) to

reconstitute the extract, and then vortexed for 1 min. The extract was filtered using 0.25 µm nylon syringe filter (Chrom Tech Inc., Apple Valley, MN, USA) into autosampler vials.

Sample preparation- QuEChERS method 2

10 ± 0.05 g of finely milled samples were weighed into a 50 mL Teflon centrifuge tube, unprocessed rice samples were used for the reagent blanks and for spiked samples. 10 mL MilliQ water and 10 mL 10% formic acid in ACN were added then the tube vortexed for 30 s. The tube was shaked with an automatic horizontal shaker at maximum speed for 1 h to fully disperse the sample, then a mixture of pre-weighted anhydrous $MgSO_4$, NaCl and citrate buffer were added and the tube were shaked immediately and vigorously by hand for 1 min. The tube was centrifuged at 3500 rpm (2180 rcf) for 5 min; for d-SPE clean-up, 2 mL of the ACN extract (upper layer) were transferred into a 2 mL centrifuge tube containing PSA (0.4 g) sorbent, then the tube was shaked vigorously by hand for 1 min and centrifuged at 3500 rpm for 5 min. 1 mL of the extract was transferred to a glass tube and evaporated to dryness at 40°C under a stream of N_2. 0.5 mL of mobile phase were added A/B (1:1, v/v) to reconstitute the extract, and then vortexed for 1 min. The extract were filtered using 0.25 µm nylon syringe filter (Chrom Tech Inc., Apple Valley, MN, USA) into autosampler vials.

Sample preparation- QuEChERS method 3

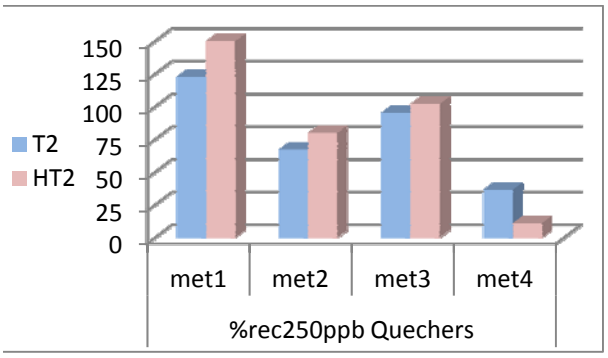
10 ± 0.05 g of finely milled samples were weighed into a 50 mL Teflon centrifuge tube, unprocessed rice samples were used for the reagent blanks and for spiked samples. 10 mL MilliQ water and 10 mL 10% acetic acid in ACN were added then the tube vortexed for 30 s. The tubes were shaked with an automatic horizontal shaker at maximum speed for 1 h to fully disperse the sample, then a mixture of pre-weighted anhydrous $MgSO_4$, NaCl and citrate buffer were added and the tube were shaked immediately and vigorously by hand for 1 min. Tubes were centrifuged at 3500 rpm (2180 rcf) for 5 min; for d-SPE clean-up, 8 mL of the ACN extract (upper layer) were transferred into a 15 mL centrifuge tube containing C18 (0.25 g) and PSA (0.4 g) sorbents, then the tubes were shaked vigorously by hand for 1 min and centrifuged at 3500 rpm for 5 min 1 mL of the extract was transferred to a glass tube and evaporated to dryness at 40°C under a stream of N_2. 0.5 mL of mobile phase were added A/B (1:1, v/v) to reconstitute the extract, and then vortexed for 1 min. The extract was filtered using 0.25 µm nylon syringe filter (Chrom Tech Inc., Apple Valley, MN, USA) into autosampler vials.

Sample preparation- QuEChERS method 4

10 ± 0.05 g of finely milled samples were weighed into a 50 mL Teflon centrifuge tube, unprocessed rice samples were used for the reagent blanks and for spiked samples. 10 mL MilliQ water and 10 mL 10% acetic acid in ACN were added then the tube vortexed for 30 s. The tube was shaked with an automatic horizontal shaker at maximum speed for 1 h to fully disperse the sample, then a mixture of pre-weighted anhydrous $MgSO_4$, NaCl and citrate buffer were added and the tube were shaked immediately and vigorously by hand for 1 min. Tubes were centrifuged at 3500 rpm (2180 rcf) for 5 min; for d-SPE clean-up, 2 mL of the ACN extract (upper layer) were transferred into a 2 mL centrifuge tube containing PSA (0.4 g) sorbent, then the tube was shaked vigorously by hand for 1 min and centrifuged at 3500 rpm for 5 min. 1 mL of the extract was transferred to a glass tube and evaporated to dryness at 40°C under a stream of N_2. 0.5 mL of mobile phase were added A/B (1:1, v/v) to reconstitute the extract, and then vortexed for 1 min. The extract were filtered using 0.25 µm nylon syringe filter (Chrom Tech Inc., Apple Valley, MN, USA) into autosampler vials.

Table 10. QuEChERS optimization – selection of appropriate extractive phase and sorbent

	% average recovery at 250 ng/g			
	met1	met2	met3	met4
T-2	122,89	67,31	**95,31**	36,65
HT-2	161,99	80,04	**102,08**	11,14



Possible causes of these losses are insufficient extraction with solvent, the use of PSA and C18 sorbents in the d-SPE format, the process of evaporating the extract to dryness, and losses during extract transferring steps.

3.3.4 Full method validation for T-2 and HT-2 toxins in feedingstuffs

Recovery and precision

The method showed a good average recovery for both the analytes (between 80% and 130%, thus meeting the performance criteria required by Law) and a good precision, with a percent variability coefficient (CV%) less than 40% both for the within day and between day repeatability at the three concentration levels. All data are showed in table 11. Good results for recovery were also evaluated at the concentration of 5 µg/Kg and 2000 µg/Kg (>70%), thus demonstrating a wide concentration range of applicability of the method developed.

Table 11. Validation results for the analysis of T-2 and HT-2 toxins in feedingstuffs

Levels (µg/Kg)	Accuracy (%) Average recovery of repeatability	Accuracy (%) Average recovery of reproducibility	Repeatability (CV%)	Reproducibility (CV%)
T-2- Na^+				
5	81.6	110.6	23.7	34.3
250	106.3	114.6	17.9	15.7
2000	98.0	120.2	19.2	35.9
HT-2-Na^+				
5	87.2	90.7	18.7	24.4
250	87.4	107.2	18.1	23.1
2000	96.8	90.7	13.7	21.8

Specificity and analysis of incurred samples

To determinate method specificity, 20 blank samples were analyzed. To evaluate the applicability of the method to all the matrix of interest, we performed analysis of representative commodities, 9 of feed samples supposed for various animal species, 3 of oat, 5 of feedingstuffs, 2 of barley and 3 of maize. Samples were prepared following the procedures described. All identification criteria (t_R, peak shape and ion ratio) were used to identify the mycotoxins in these samples. Overall results are summarized in table 12, while in figure 6 are shown an incurred barley sample (barley 2, B) both for T-2 and HT-2 toxins compared with a 5 µg/Kg spiked sample.

Figure 6. Presence of T-2 and HT-2 toxins in feedingstuffs

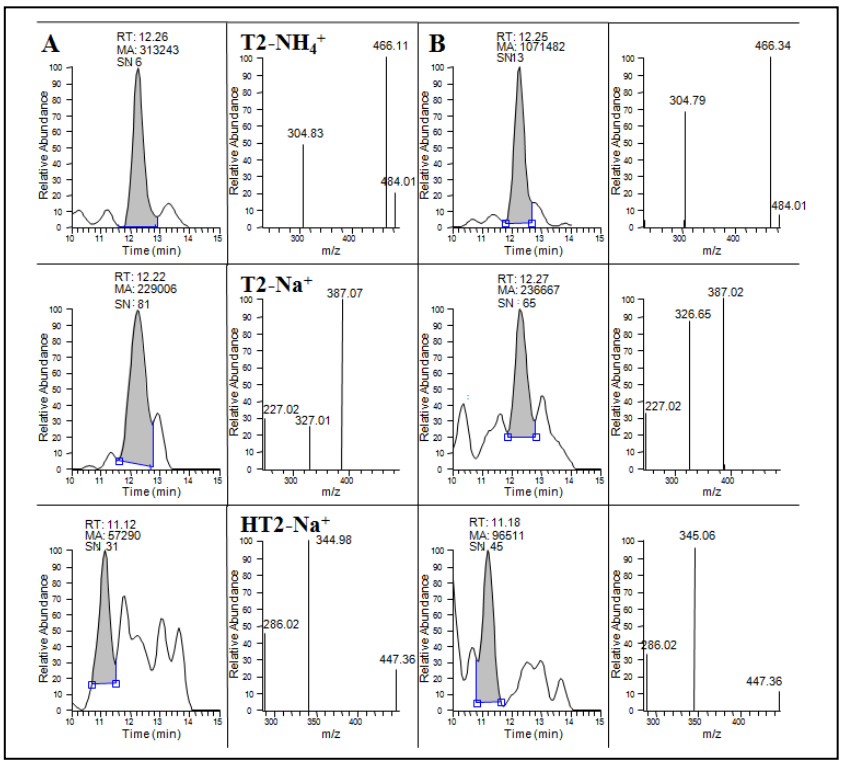


Table 12. Analysis of real feedingstuffs samples

	Concentration (µg/Kg)	
SAMPLE	T-2 toxin	HT-2 toxin
Oat1	nr	nr
Oat2	nr	nr
Oat3	5.6	nr
Barley1	nr	nr
Barley2	10.9	11.4
Cattle feedingstuffs 1	18.2	nr
Cattle feedingstuffs 2	nr	nr
Pig feedingstuffs 1	5.0	nr
Pig feedingstuffs 2	nr	nr
Maize 1	nr	nr
Maize 2	nr	nr
Maize 3	nr	nr
Broiler feedingstuffs 1	nr	nr
Broiler feedingstuffs 2	nr	nr
Broiler feedingstuffs 3	8.1	nr
Hen feedingstuffs	nr	nr
Rabbit feedingstuffs	5.0	nr

Method validation - Robustness trial

To evaluate method robustness, we chose 7 parameters that may affect the outcome of the extraction and assigned the modified values to them as follows:

Table 13. Method value and modified value in the robustness trial

	Variabile	**Method value**	**Modified value**
Parameter A	Volume of water with which sample is initially treated	10 mL	9 mL
Parameter B	Concentration of acetic acid in the acetonitrile in the extraction step	10%	9%
Parameter C	Soaking time in the extraction step	60 min	50 min
Parameter D	Salt mix volume for the salt-out step	4 g	3.5 g
Parameter E	Centifugation time after soaking step	5 min	4 min
Parameter F	Volume of the extracted sample treated with dispersive PSA/C18	8 mL	6 mL
Parameter G	Centifugation time after clan-up step	5 min	4 min

After, we conducted the 8 runs over spiked samples at the concentration of 250 µg/Kg utilizing the specific combinations of standard and modified values for the factors as follows, and recorded the result obtained for each combination. It is essential that the factors be combined exactly as specified or erroneous conclusions will be drawn.

Table 14. Run factor combinations measurement

Runs		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
		A	A	A	A	a	a	a	a
		B	B	b	b	B	B	b	b
		C	c	C	c	C	c	C	c
		D	D	d	d	d	d	D	D
		E	e	E	e	e	E	e	E
		F	f	f	F	F	f	f	F
		G	g	g	G	g	G	G	g
Results obtained in terms of concentration (µg/Kg)	**T-2**	258.6	228.2	273.2	283.7	280.1	292.4	279.3	279.7
	HT-2	182.4	191.8	209.4	157.9	211.8	205.6	215.7	209.6
Results obtained in terms of % recovery	**T-2**	103.4	91.3	109.3	113.5	112.0	117.0	111.7	111.9
	HT-2	73.0	76.7	83.7	63.2	84.7	82.2	86.3	83.8

To obtain the effect of each of the factors, the differences of the measurements containing the subgroups of each variation was calculated as described in the section material and methods, then a standard deviation of the differences D_i (S_{Di}).

Factor		**D_A**	**D_B**	**D_C**	**D_D**	**D_E**	**D_F**	**D_G**
Standard deviations (µg/Kg)	**T-2**	21.9	14.1	1.8	20.9	8.1	7.2	13.2
	HT-2	25.3	0.22	13.6	3.7	7.4	15.2	15.2

The S_{Di} were 20.1 µg/Kg for T-2 toxin and 19.7 µg/Kg for HT-2 toxin. The values were compared with the reproducibility standard deviation S_r (inter die) at the same spiking level, being 44.9 µg/Kg for T-2 toxin and 62.0 µg/Kg for HT-2 toxin.

3.4 Conclusions 1

The analytical method for the determination of T-2 toxin and HT-2 toxin described above have been developed and optimized with a QuEChERS extraction methodology and detection with HPLC-ESI-Ion Trap MS/MS spectrometry technique. To our scopes, method was developed for feedingstuffs and non-processed cereals such as oat, barley, maize and rye intended for animals feeding. Accurate quantification was carried out even if complex matrices as feedingstuffs and the resulting signal suppression effects owing to matrix effects have required the careful optimization of the usage of matrix matched standards. Our new method is simple, and is more time- and cost-effective compared to classical IAC methods. The method was validated according to guidelines and met acceptability criteria in all cases. Good analytical results were obtained, including recovery, precision (repeatability, within- and inter-laboratory reproducibility), linearity, and analytical limits (LOD and LOQ). The method can be used to replace the IAC for routine T-2 and HT-2 mycotoxin analysis in feedingstuffs for export and some related matrices (feed, maize, oat and barley).
The method have been applied to the analysis of various real samples revealing a low concentration ranging between 5 and 20 μg/Kg, thus demonstrating a low sanitary risk for animal health.

3.5 References

Commission Regulation No. 657/2002 of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Official Journal of the European Union, L221, 2002, pp. 8–36

Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs.

Youden WJ & Steiner EH. Statistical Manual of the Association of Official Analytical Chemists, 1975, pp. 50-55

3.6 Topic 2 - Development and validation of screening and confirmatory methods for the analysis of cyanotoxins in dietary cyanobacterial and algal supplements

The aim of the study was to develop and, possibly, validate methods for the determination of cyanotoxins in dietary supplements with a mass spectrometry approach. In particular, we focused our attention on microcystins and cylindrospermopsin. To do this they have been employed methods consisting in an extraction step with acidified organic solvent/water followed by a clean-up step in classic SPE. They were used to prepare blank and artificially spiked samples. Extracted samples were then analyzed in LC-ESI-ion trap technique with different chromatographic separation and mass spectrometric detection. Series of experiments allowed us to evaluate method performances and, eventually, validate a confirmatory method for the determination of microcystins in cyanobacterial and algae dietary supplements.

3.7 Matherials and methods 2

3.7.1 Chemicals and reagents

Microcystin-LR (MC-LR), Microcystin-YR (MC-YR), Microcystin-RR (MC-RR), Microcystin-des-Methyl-LR (des-Me-MC-LR) and Microcystin-des-Methyl-RR (des-Me-MC-RR), were supplied by DHI Water and Environment (Denmark); Microcystin-LF (MC-LF) and Microcystin-LW (MC-LW) were producted by ALEXIS (ALEXIS corporation, Lausen, Swizerland) and supplied by Vinci Biochem (Vinci, Italy) in screw capped vials containing 25 µg standard. All the reference materials were of analytical grade purity. Cylindrospermopsin reference material standard was supplied by NRC (National Research Council, Canada) and was of analytical grade purity, it was purchased in standard stock solutions in MilliQ water, at 12.4 µg/mL with a vial content of 0.5 mL and it was stable for 1 year if stored at 4°C.
HPLC grade acetonitrile, methanol, *n*-exane, glacial acetic acid (99%) and trifluoroacetic acid were from Carlo Erba (Milan, Italy). HPLC grade water was produced using a MilliQ system (Millipore, Bedford, MA, USA).
C18 end-capped (EC) solid-phase extraction (SPE) cartridges with 1 g sorbent bed and 6 mL reservoir volume (Isolute, UK) were purchased from Step-Bio (Bologna, Italy), HLB-Oasis solid-phase extraction (SPE) cartridges with 0.5 g sorbent bed and 6 mL reservoir volume were supplied by Waters (Waters, Milford, MA, USA). The Centrifuge Filters Microsep 100K OMEGA 100 KDa cut-off were from PALL Life Science (Pall Corporation, Port Washington, NY, USA).
The ESI-Ion Trap mass spectrometer was periodically calibrated with standard solutions of Ultramark, caffeine and Met-Arg-Phe-Ala peptide provided by the manufacturer (Thermo Fisher).

3.7.2 Apparatus – ESI ion-trap

Analysis were performed using a LC/ESI-MS/MS system equipped with a Surveyor LC Pump Plus (Quaternary Pump), a Surveyor Autosampler Plus and a LCQ Advantage ion trap mass spectrometer with an ESI source (ThermoFisher Scientific). The mass spectrometer was periodically calibrated with solutions of Ultramark, caffeine and Met-Arg-Phe-Ala peptide provided by the manufacturer (ThermoFisher Scientific).
The data were acquired and processed using the Xcalibur™ software, version 1.3, from ThermoElectron.

3.7.3 Reverse phase HPLC separation of desMe-MC-LR, MC-LR, MC-YR MC-LW and MC-LF

Chromatographic separation was performed by injecting 50 µL of samples onto a 250 × 3.00 mm Synergi Max RP 80 Å 4 µm particle stainless steel column (Phenomenex, Torrance, CA, USA), at 0.3 mL/min, using 0.05 % TFA in MilliQ water as mobile phase A and 0.05 % TFA in acetonitrile as mobile phase B, the elution at room temperature was performed under the following conditions: from 30 to 100% B in 20 minutes, then held at 100% B for the next 5 minutes; returned to 30% over 2 minutes, then held for another 10 minutes.

3.7.4 Reverse phase HPLC separation of desMe-MC-RR, desMe-MC-LR, MC-RR, MC-LR and MC-YR

Chromatographic separation was performed by injecting 50 µL of samples onto a 150 × 3.00 mm Luna PFP 5 µm particle stainless steel column (Phenomenex, Torrance, CA, USA), at 0.6 mL/min, using 0.05 % TFA in MilliQ water as mobile phase A and 0.05 % TFA in acetonitrile as mobile phase B, the elution at room temperature was performed under the following conditions: 5 % B held from 0 to 2 minutes, then from 5 % B to 100% B in 10 minutes, held at 100 % B for 2 minutes; returned to 5 % B over 3 minutes, then held for another 5 minutes.

3.7.5 ESI ion-trap analysis of MCCs in the negative ion mode

The mass spectrometer was periodically calibrated with solutions of Ultramark, caffeine and Met-Arg-Phe-Ala peptide provided by the manufacturer. During the LC/ESI-MS/MS experiments, mass spectra were acquired in the negative ion mode for five of the seven MCCs. The instrument was daily calibrated for the analysis by tuning on des-Me-MC-LR molecular ion $[M-H]^-$ m/z 979.4, on MC-LR molecular ion $[M-H]^-$ m/z 993.3 and on MC-LW molecular ion $[M-H]^-$ m/z 1023.5.
The LC/ESI-MS/MS analysis was performed by monitoring the signals of the precursor ion → product ion transitions (selected reaction monitoring, SRM mode) from the LC/ESI-MS/MS dataset of each MCC. The diagnostic ions for MS/MS qualitative analysis are reported in Table 14.

Table 14. The experimental conditions and selected diagnostic ions for the Ion Trap LC/ESI-MS/MS analysis of MCCs in the negative ion mode

	MS/MS conditions				
	MC-YR	MC-LR	desMe-MC-LR	MC-LW	MC-LF
Ionization mode	negative	negative	negative	negative	negative
Maximum injection time (ms)	300	300	300	300	300
Isolation width (m/z)	2	2	2	2	2
Molecular ion $[M-H]^-$(*m/z*)	1043.4	993.3	979.4	1023.5	984.5
MS/MS precursor ion $[M-H]^-$ (*m/z*)	1043.4	993.3	979.4	1023.5	984.5
Collision energy (%)	32	32	32	35	35
Selected diagnostic MS/MS ions (*m/z*) (SRM)	1043.4 1025.3 931.4	993.3 975.4 962.4	979.4 961.3 904.3	1023.5 1005.3 992.3	984.5 966.3 953.3
Selected diagnostic MS/MS ions (*m/z*) Quantitative analysis	1025.3	975.4	961.3	1005.3	966.3

3.7.6 ESI ion-trap analysis of MCCs in the positive ion mode

For MCCs analysis in the positive ion mode the instrument was daily calibrated for the analysis by tuning on des-Me-MC-RR molecular ion $[M+H]^+$ m/z 1024.3 and on MC-LR molecular ion $[M+H]^+$ m/z 995.3.
The LC/ESI-MS/MS analysis was performed by monitoring the signals of the precursor ion → product ion transitions (selected reaction monitoring, SRM mode) from the LC/ESI-MS/MS dataset of each MCC. The diagnostic ions for MS/MS qualitative analysis are reported in Table 15.

Table 15. The experimental conditions and selected diagnostic ions for the ion trap LC/ESI-MS/MS analysis of MCCs in the positive ion mode

	MS/MS conditions				
	desMe-MC-LR	MC-RR	desMe-MC-RR	MC-LR	MC-YR
Ionization mode	positive	positive	positive	positive	positive
Maximum injection time (ms)	300	300	300	300	300
Isolation width (m/z)	2	2	2	2	2
Molecular ion $[M+H]^+$ (*m/z*)	981.0	1038.5	1024.3	995.3	1045.4
MS/MS precursor ion $[M+H]^+$ (*m/z*)	981.0	1038.5	1024.3	995.3	1045.4
Collision energy (%)	35	35	35	35	35

	981.0	1038.5	1024.5	995.3	1045.4
Selected diagnostic MS/MS ions	953.3	1020.4	1006.4	977.6	1027.3
(*m/z*) (SRM)	599.2	996.2	982.2	599.2	599.2
		909.5	909.4		
Selected diagnostic MS/MS ions (*m/z*) Quantitative analysis	953.3	1020.4	1006.4	977.6	1027.3

3.7.7 LC-ESI ion-trap analysis of CYN

Chromatographic separation was performed with gradient elution, at 20°C, injecting 50 µL sample volume on a 3 µm particle 150 × 3.0 mm Luna PFP RP 80 Å Synergi stainless steel column (Phenomenex, Torrance, CA, USA), at 0.25 mL/min flow rate, using 0,1% acetic acid in water as mobile phase A, and 0,1% acetic acid in methanol as mobile phase B, the elution at room temperature was performed under the following conditions: 5 % B held from 0 to 2 minutes, then from 5 % B to 20% B in 8 minutes, held at 100 % B for 2 minutes; returned to 5 % B over 3 minutes, then held for another 5 minutes.
During the LC/ESI-MS/MS experiments, mass spectra were acquired in the positive ionization mode; the spectrometer parameters were optimized by tuning on the $[M+H]^+$ ion of CYN, at *m/z* 416.2. Tuning was performed at 0.25 mL/min LC flow rate. The following experimental LC/ESI-MS/MS parameters were set: capillary temperature 300 °C, spray voltage 4.5 kV, microscan number 3, maximum inject time 200 ms. Further experimental conditions (collision energy, isolation width), and the diagnostic ions for MS/MS qualitative analysis, are also reported (Table 16). The LC/ESI-MS/MS analysis was performed by monitoring the signals of the precursor ion → product ion transitions (selected reaction monitoring, SRM mode) from the LC/ESI-MS/MS dataset.

Table 16. The experimental conditions and selected diagnostic ions for the ion trap LC/ESI-MS/MS analysis of CYN

	MS/MS conditions
Ionization mode	positive
Maximum injection time (ms)	200
Isolation width (m/z)	2
Molecular ion $[M+H]^+$(*m/z*)	416.2
Collision energy (%)	35
Selected diagnostic MS/MS ions (*m/z*) (SRM)	416.2→ 336.2 416.2→ 318.2 416.2→ 274.1
Selected diagnostic MS/MS ions (*m/z*) Quantitative analysis	336.2

3.7.8 Standard solutions of MCCs

MC-RR and MC-LR reference materials were purchased in at 10 µg/mL in methanol, while MC-YR standard stock solutions were at 6.6 µg/mL in methanol, desMe-MC-RR standard stock solutions were at 8.1 µg/mL in methanol and des-Me-MC-LR standard stock solutions were at 7.1 µg/mL in methanol. To prepare the standard stock solutions of MC-LF and MC-LW at 10 µg/mL, the vial content of each compound was dissolved in 2.5 mL of methanol. A standard mix solution of all the seven molecules at 1.0 µg/mL was prepared by mixing the appropriate volumes of each single reference material solution and diluting with methanol. In matrix working solutions at 5, 10, 20, 50, 75 and 100 ng/mL (corresponding to 25, 50, 100, 250, 375 and 500 ng/g in the sample) were daily prepared by diluting standard mix solution of all the seven molecules at 1.0 µg/mL with an uncontaminated extracted and cleaned-up sample.

3.7.9 Standard solutions of cylindrospermopsin

To prepare CYN an intermediate working solution at 0.5 µg/mL, 100 µL of the standard stock solutions in MilliQ water, at 12.4 µg/mL was diluted with methanol to 0.5 mL, the working standard solutions at 10, 25, 50, 75, 100 and

200 ng/mL were prepared daily from the working solution at 0.5 μg/mL by diluting with methanol. In matrix working solutions at 5, 10, 25, 50, 75 and 100 ng/mL (corresponding to 50, 100, 200, 500, 750 and 1000 ng/g in the sample) were daily prepared by diluting standard solution of 0.5 μg/mL with an uncontaminated extracted and cleaned-up sample.

3.7.10 Algae food supplement samples extraction and clean up for analysis of MCCs

0.3 g of sample was weighted into 10 mL glass centrifuge tube and 3 mL of 0.1 % trifluoroacetic acid in 1/3 v/v methanol/MilliQ water were added and the mixture vortexed for at least 30 seconds, then the mixture was treated for 10 minutes using ultrasonication and centrifuged at 1086 *g* for 15 minutes. 2 mL of the supernatant were transferred into an eppendorf tube and centrifuged at 18790 *g* for 15 minutes then collected and transferred to Centrifuge Filters Microsep 100K and filtrated by centrifugation at 2000 *g* for 30 minutes. The filtrated was made up to a final volume of 20 mL with MilliQ water and the clean-up was performed by loading onto the C18 EC SPE column preventively conditioned with 5 mL methanol, followed by 5 mL of MilliQ water. The solution was passed through the column at a flow rate of approximately 1 mL/min and the column was washed with 5 mL of methanol/water 10/90 v/v, the column was then dried for 30 minutes. The column was finally eluted with 5 mL TFA 1% methanol, dried under a nitrogen stream at 40°C, then dissolved in 0.5 mL methanol and analyzed by LC/ESI-MS/MS; spiked samples with a concentration higher than 500 ng/g were opportunely diluted before analysis.

3.7.11 Algae food supplement samples extraction and clean up for analysis of CYN

0.5 g of sample was weighted into 10 mL glass centrifuge tube and 5 mL of 0.05 % acetic acid in 1/3 v/v MilliQ/Acetonitrile was added and vortexed for at least 30 seconds, then the mixture was treated for 10 minutes using ultrasonication and centrifuged at 1086 *g* for 15 minutes. 2 mL of the supernatant were transferred into an eppendorf tube and centrifuged at 18790 *g* for 15 minutes then collected and transferred to Centrifuge Filters Microsep 100K and filtrated by centrifugation at 2000 *g* for 30 minutes. The filtrated was made up to a final volume of 20 mL with MilliQ water and the clean-up was performed by loading onto the HLB-Oasis SPE column preventively conditioned with 5 mL methanol, followed by 5 mL of MilliQ water. The solution was passed through the column by gravity and the column was washed with 5 mL of methanol/water 10/90 v/v, the column was then dried for 30 minutes. The column was finally eluted with 5 mL acetic acid 0.05% methanol, dried under a nitrogen stream at 40°C, then dissolved in 0.5 mL methanol and analyzed by ion trap LC/ESI-MS/MS; spiked samples with a concentration higher than 500 ng/g were opportunely diluted before analysis.

3.8 Results and discussion 2

3.8.1 LC-ESI-Ion Trap analysis of MCCs in the negative ion mode

A method previousely developed in our laboratories (Gallo et al, 2012) was applied for the analysis of microcystins in algae dietary supplements. The seven MCCs were searched in the extracted and cleaned-up samples in a qualitative and quantitative determination with the technique based upon a separation in liquid chromatography with a gradient programme coupled with mass spectrometry Ion-trap LC/ESI-MS/MS in the negative ion mode.
The method had already been tested in terms of repeatability, reproducibility at the level of 100 ng/g. Now, we wanted to develop a complete validation study and apply it for the analysis of real algae dietary samples. To do this, several method performances were evaluated an herein reported: accuracy, precision in terms of repeatability, reproducibility, applicability range; linearity and matrix effects were also evaluated. The qualitative determination was also evaluated with MS/MS spectra comparison with identification point criterion. With our study we were able to demonstrate that this method had good performances for a complete validation for the following molecules: MC-LW, MC-LF and desMeMC-LR, while other LC/ESI-MS/MS methods were developed and validated to analyze MC-RR, MC-LR, MC-YR and desMeMC-RR.

3.8.2 Linearity and matrix effects for MC-LW, MC-LF and desMeMC-LR

The linearity of response vs. concentration was evaluated using 7 matrix-matched calibration points (not including zero concentration) over the range of 5–200 ng/mL. All analytes gave average linear regression (R^2) values of >0.95 over the range of interest (Table 17).
Matrix effects were unavoidable and we were not able to eliminate them in the analysis. Table 17 shows our estimated matrix effects, calculated from the differences between the matrix-matched standards and solvent-based standards calibrations. MC-LW, MC-LF and desMeMC-LR, that were ionized in negative mode showed similar trends of signal enhancement (up to 50%). Moreover, matrix effects seemed to be independent on concentration. All these results indicated the necessity of using matrix-matched standards to quantify analytes, in order to compensate for matrix effects.

Table 17. Retention times (t_R) with a tolerance range, linear regressions (R^2) in matrix, matrix effects, and analytical limits for the 3 microcystins analyzed with the negative ion mode.

Analyte	Ionization mode	t_R ± tolerance value (min)	linear regressions (R^2) in matrix	matrix effects (%)	analytical limits (ng/g)
desMeMC-LR	negative	14.54 ± 0.36	0.971	64	50
MC-LW	negative	20.25 ± 0.51	0.989	75	50
MC-LF	negative	20.77 ± 0.52	0.965	142	50

3.8.3 Full method validation data for MC-LW, MC-LF and desMeMC-LR

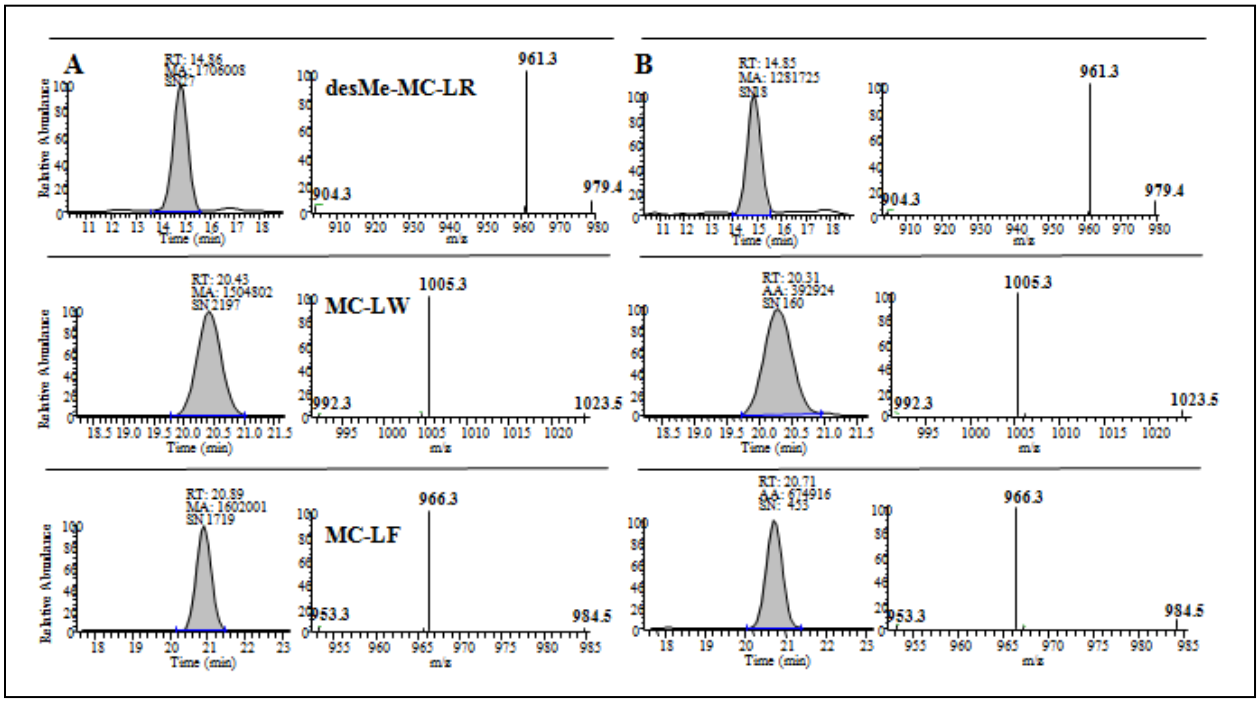
Results indicated that the three substances MC-LW, MC-LF and desMeMC-LR were unambiguously identified with the identification points criterion, with at least the presence of 3 diagnostic ions (1 precursor and 2 product ions) thus showing the high specificity of the method. The method also shows a good average recovery for the three analytes (>70%) and a good precision, with a percent variability coefficient (CV%) less than 30% both for the within day and between day repeatability at the concentration range of 100 ng/g. All data are showed in table 18. Good results for recovery were also evaluated at the concentration of 50 ng/g and 2000 ng/g (>70%), thus demonstrating a wide concentration range of applicability of the method developed. The observed enhancement of the signal eventually improves the signal to noise ratio and so the method sensibility. Overall results are showed in table 18.

Table 18. Validation results for the analysis of MC-LW, MC-LF and desMeMC-LR in algae dietary supplements samples

Levels (ng/g)	Accuracy (%) Average recovery of repeatability	Accuracy (%) Average recovery of reproducibility	Repeatability (CV%)	Reproducibility (CV%)
desMe-MC-LR				
50	107.3	129.4	24.2	24.8
100*	95.6	102.7	11.6	12.8
2000	97.3	84.0	23.4	26.3
MC-LW				
50	114.8	119.8	7.3	12.1
100*	76.1	72.0	2.8	10.5
2000	102.4	75.5	8.9	26.8
MC-LF				
50	108.3	99.5	8.3	28.6
100*	77.0	75.0	12.7	11.2
2000	81.2	78.4	14.9	14.0

*Data obtained in a previous study (Gallo et al, 2012)

Figure 7. The SRM Chromatograms of an in matrix standard of the 3 molecules analyzed (A) and 100 ng/g recovery (B) from dietary supplements. For each molecule are also reported the MS/MS spectra.



3.8.4 MC-LR and MC-YR in the negative ion mode

The same experiments carried out for MC-LW, MC-LF and desMeMC-LR were carried out for MC-LR and MC-YR. Results showed that for these two molecules we were not able to assess a limit of quantification (LOQ) at the concentration level of 50 ng/mL, thus confirming results previously obtained in our laboratories, i.e. a LOQ of 100 ng/mL. Results also indicate that with this method the two substances were not always unambiguously identified with the identification points criterion, with at least the presence of 3 diagnostic ions (1 precursor and 2 product ions). So the analytical method for the detection of these two congeners needed to be improved. To do that, we develop another chromatographic separation with a Luna PFP column, thus improving peak sharpness and the signal to noise ratio. We find the negative mode as the best polarity to develop the quantitative analysis, even if, for unambiguous identification, extra analysis were performed with the same chromatographic conditions, but in the positive ion mode. With this new chromatographic separation, we were able to extimate matrix effects (a signal suppression was observed for both the molecules), to calculate a repeatability over 6 replicates and reproducibility

over two sets of 6 replacates along with recovery percentage at the concentration level of 100 and 2000 ng/mL. Results are summarized in tables 19 and 20.

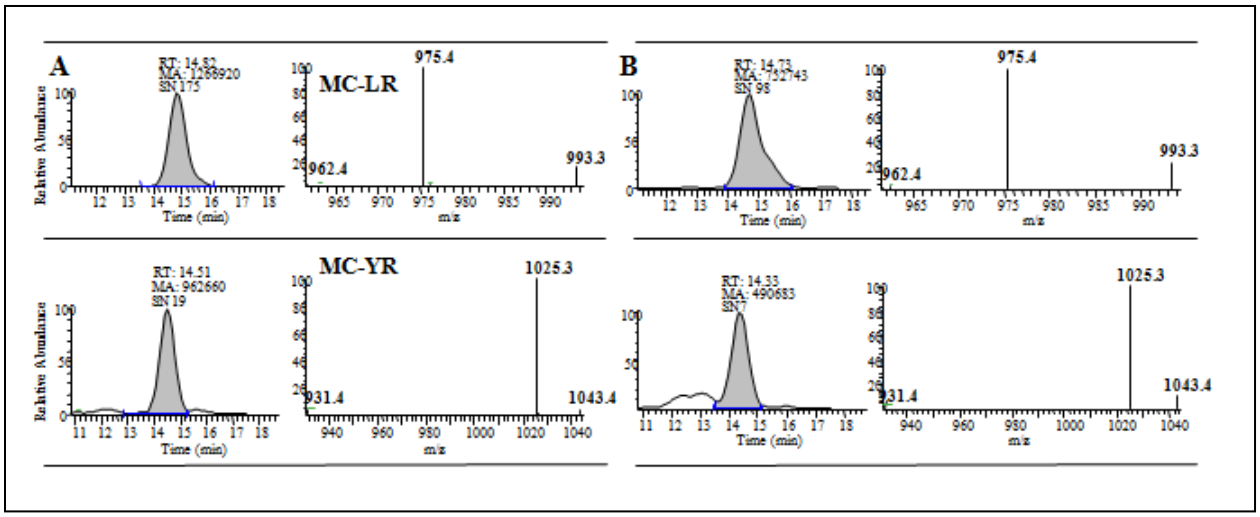
Table 19. Retention times (t_R) with a tolerance range, linear regressions (R^2) in matrix, matrix effects, and analytical limits for MC-LR and MC-YR.

Analyte	Ionization mode	$t_R \pm$ tolerance value (min)	linear regressions (R^2) in matrix	matrix effects (%)	analytical limits (ng/g)
MC-LR	negative	9.87 ± 0.25	0.982	-27%	100
MC-YR	negative	9.77 ± 0.24	0.972	-128%	100

Table 20. Validation results for the analysis of MC-LR and MC-YR in algae dietary supplements samples

Levels (ng/g)	Accuracy (%) Average recovery of repeatability	Accuracy (%) Average recovery of reproducibility	Repeatability (CV%)	Reproducibility (CV%)
MC-LR				
100	82.2	88.5	27.5	27.9
2000	86.4	87.5	17.5	23.1
MC-YR				
100	113.2	119.9	28.7	24.9
2000	119.1	103.1	9.4	22.5

Figure 8. The SRM Chromatograms in the negative ion mode of an in matrix standard of the MC-YR and MC-LR (A) and 100 ng/g recovery (B) from dietary supplements. For each molecule are also reported the MS/MS spectra.



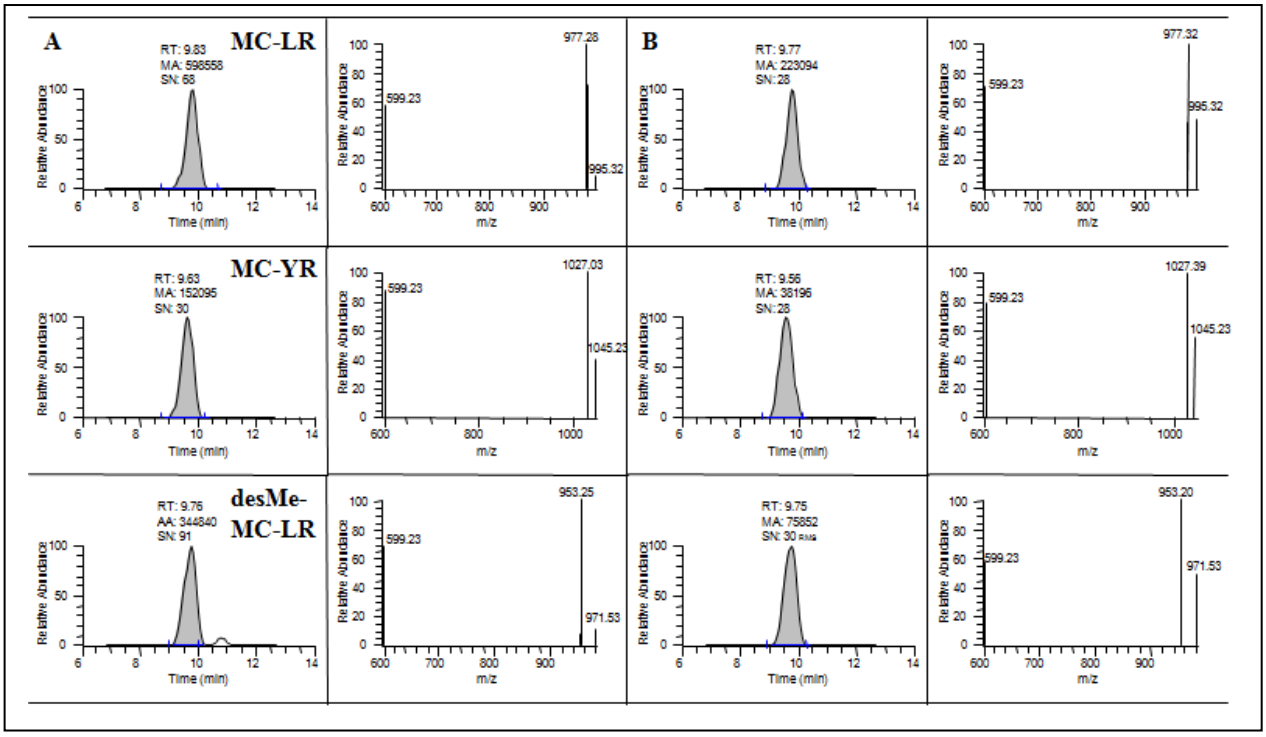
3.8.5 LC-ESI-Ion Trap analysis of MCCs in the positive ion mode

MC-RR, MC-LR, MC-YR, desMeMC-LR and desMeMC-RR were searched in the extracted and cleaned-up samples in a qualitative and quantitative determination with a technique based upon a separation in liquid chromatography onto a Luna PFP column with a gradient programme and detection with mass spectrometry ESI Ion-trap in the positive ion mode. This method was the best choice for MC-RR and desMeMC-RR variants both for quantitative and qualitative detection, while MC-LR, desMeMC-LR and MC-YR, as said, needed extra identification points for unambiguous identification.

In this work, to carry out a complete validation study for MC-RR and desMe-MC-RR we tested a serie of performance method parameters: accuracy, precision in terms of repeatability and reproducibility. applicability range; linearity and matrix effects were also evaluated. The qualitative determination was also evaluated with MS/MS spectra comparison with identification point criterion. This method showed good performances for MC-RR and desMeMC-RR; for MC-LR, desMeMC-LR and MC-YR we were able to separate and identificate the molecules down to 100 ng/mL, thus giving extra identification points needed for confirmation analysis and successfully used later for the analysis of real samples. Because of the bias effects, the best choice to extract the chromatograms in the

positive ion mode for MC-LR, desMeMC-LR and MC-YR was the transition [M+H] → 599.2 (a daughter ion common to all the microcystins).

Figure 9. The SRM Chromatograms in the positive ion mode of an in matrix standard of the MC-LR, MC-YR and desMeMC-LR (A) and 100 ng/g recovery (B) from dietary supplements. For each molecule are also reported the MS/MS spectra.



3.8.6 MC-RR and desMe-MC-RR - linearity, matrix effects and full validation results

The linearity of response vs. concentration was evaluated in a wide concentration range using 7 matrix-matched calibration points (not including zero concentration) over the range of 5–200 ng/mL. Both MC-RR and desMeMC-RR had good average linear regression (R^2) values over the range of interest (Table 21).
Even in this case matrix effects were unavoidable. Table 21 shows our estimated matrix effects, calculated from the differences between the matrix-matched standards and solvent-based standards calibrations. Both MC-RR and desMeMC-RR showed a signal suppression. Moreover, matrix effects seemed to be independent on concentration.
All these results indicated the necessity of using matrix-matched standards to quantify analytes, in order to compensate for matrix effects.

Table 21. Retention times (t_R) with a tolerance range, linear regressions (R^2) in matrix, matrix effects, and analytical limits for desMeMC-RR and MC-RR.

Analyte	Ionization mode	t_R ± tolerance value (min)	linear regressions (R^2) in matrix	matrix effects (%)	analytical limits (ng/g)
desMeMC-RR	positive	9.24 ± 0.23	0.997	-16%	50
MC-RR	positive	9.33 ± 0.23	0.986	-36%	50

Results indicated that the two substances were unambiguously identified with the identification points criterion, the presence of 4 diagnostic ions (1 precursor and 3 product ions) thus showing the high specificity of the method. The method also shows a good average recovery at the contamination level of 50 ng/g (> 80 %) and a good precision, with a repeatability and reproducibility CV% at the concentration of 50 ng/g showed in table 22. Repeatability and reproducibility were also evaluated at the concentration of 100 and 2000 ng/g to demonstrate the range of applicability of the method developed.

Figure 10. The SRM Chromatograms of an in matrix standard of the MC-RR and desMeMC-RR (A) and 50 ng/g recovery (B) from dietary supplements. For each molecule are also reported the MS/MS spectra.

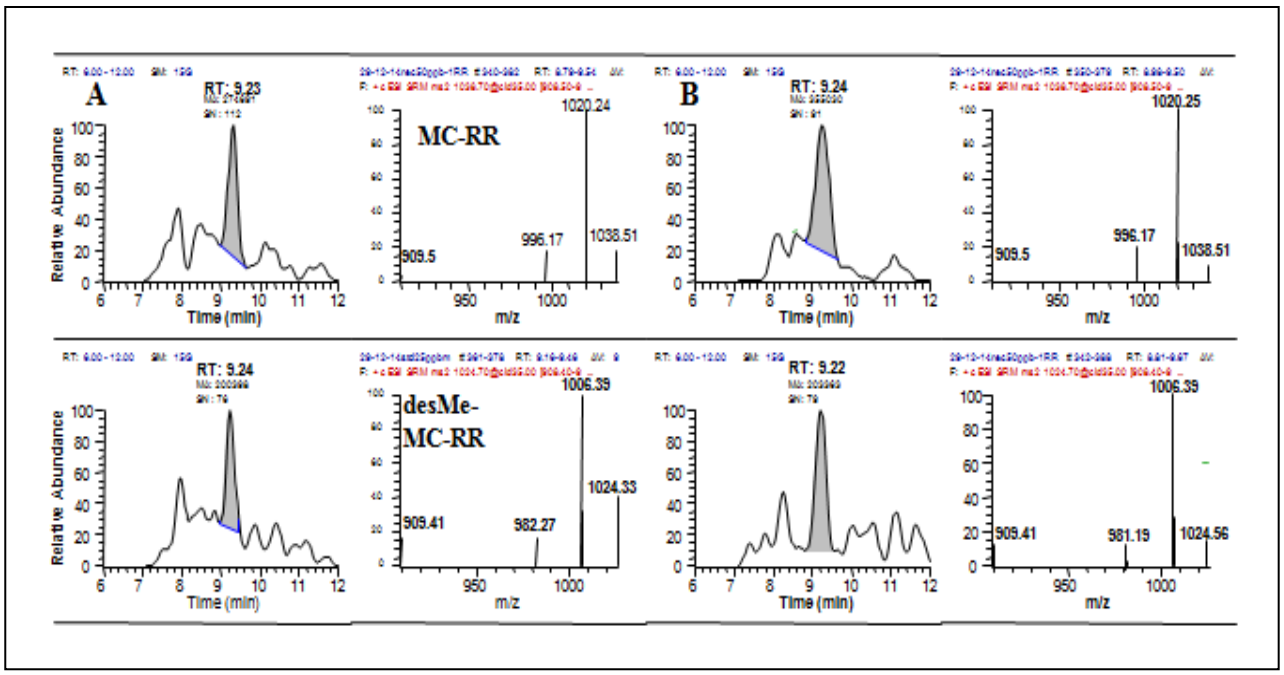


Table 22. Method performances for desMeMC-RR and MC-RR in the positive ion mode

Levels (ng/g)	Accuracy (%) Average recovery of repeatability	Accuracy (%) Average recovery of reproducibility	Repeatability (CV%)	Reproducibility (CV%)
desMe-MC-RR				
50	89.0	97.9	28.4	30.8
100	87.3	99.3	20.5	25.0
2000	101.1	101.4	12.8	18.0
MC-RR				
50	87.4	88.2	15.1	16.0
100	111.4	101.5	12.7	21.0
2000	94.3	83.5	5.6	15.5

3.9 Conclusions 2

A new method for the separation and detection of seven microcystins from a complex matrix is described. The results of our work demonstrated that the classic SPE separation provides a fast and simple way to isolate the microcystins from dietary supplements and liquid chromatography coupled with mass spectrometry ESI ion trap offers a reliable detection method even if this mass analyzer is a less expensive technique than others such as the triple quadrupole, which is the mostly adopted for this kind of analysis (Bruno et al, 2006, Gallo et al, 2012, Heussner et al, 2012).

The method was developed on artificially spiked samples and successfully tested in terms of specificity, accuracy and non ambiguous qualitative detection, it was applied to real samples of dietary algae and cyanobacterial supplements sold in Italy without prescription and some of them resulted contaminated. There are no previous works reporting analysis of MCCs from dietary supplements with LC/ESI ion trap technique.

As to cylindrospermopsin detection, a new method with LC-ESI-ion trap technique was developed.

These two methods were then successfully used for the analysis of cyanotoxin in real dietary supplement samples and results are described in the next section.

3.10 References

Bruno M, Fiori M, Mattei D, Melchiorre S, Messineo V, Volpi F, Bogialli S, Nazzari M (2006) ELISA and LC-MS/MS methods for determining cyanobacterial toxins in blue-green algae food supplements. Nat Prod Res 20:827-34.

Gallo P, Nitride C, Nasi A, Gualtieri L, Ferranti P. (2012) Monitoring of algal biotoxins in sea products and dietary supplements: development of an analytical method to ensure food quality and safety. Ingredienti alimentari. Chiriotti editore 64:6-11.

Heussner AH, Mazija L, Fastner J, Dietrich DR (2012) Toxin content and cytotoxicity of algal dietary supplements. Toxicology and Applied Pharmacology 265:263-71.

3.11 Topic 3 - Monitoring the presence of cyanotoxins in dietary cyanobacterial and algae supplements

The aim of the study was to evaluate the presence of microcystins and cylindrospermopsin in algae dietary supplements. To do this they have been employed the methods described in topic 2 section. In this study the analysis for the presence of cyanobacterial MCCs encompassed only algae dietary supplements marketed in Italy and thus only the three main types of algae generally used for the production of dietary supplements, i.e. *Aph. flos-aquae*, *Spirulina* and *Chlorella*, thereby restricting the interpretation of the findings to these type of products. Eleven samples were analyzed for seven microcystins, employing the method in the negative ion mode for desMe-MC-LR, MC-LW and MC-LF, the method in the positive ion mode for desMe-MC-RR and MC-RR; whereas to confirm the presence of MC-LR both methods were needed. As to evaluation of the occurrence of cylindrospermopsin in these eleven dietary supplements, the method developed allowed us to do a screening but, due to strong matrix effects, without assessing a precise quantitative determination.

3.12 Matherials and methods 3

3.12.1 Algae dietary supplements

A total of eleven products of algae and cyanobacterial dietary supplements including tablets, powder and capsules of different brands available on the Italian market or on Internet were examinated. Their ingredient composition was variable and not all were only algal or cyanobacterial extracts. According to the description of the manifacturer, the main ingredient of the food supplements were summarized in the following table:

Table 23. Cyanobacterial and algae dietary supplements

LIST OF SAMPLES		
SAMPLE	PRODUCER	ALGAE CONTENT
SPIRULINA **Spir01**	SOLGAR	*Spirulina Platensis*
SPIRULINA **Spir02**	ALTA NATURA	*Spirulina Maxima*
SPIRULINA **Spir03**	RODIOLA	*Spirulina Maxima*
SPIRULINA **Spir04**	OPTIMA NATURALS	*Spirulina Maxima*
NUTRASPIRULINA **Spir05**	NUTRALINE	*Spirulina*
ARTHROSPIRA **Spir06**	No brand	*Spirulina Maxima*
ALGA KLAMATH AFA **Apha01**	ERBA VITA	*Aphanizomenon Flos-Aquae*
MICROALGHE KLAMATH **Apha02**	RODIOLA	*Aphanizomenon Flos-Aquae*
DIGEVIT **Apha03**	ALGOVIT	*Aphanizomenon Flos-Aquae* (4.2%)
CHLORELLA **Chlo01**	ISWARI	*Chlorella*
VITAMINA B12 & CLORELLA **Chlo02**	ERBAVOGLIO	*Chlorella*

All these dietary supplements were analyzed both for microcystins and cylindrospermopsin determination with the methodologies described in the previous section.

3.13 Results 3

3.13.1 Determination of MCCs in algae dietary supplements with LC-ESI-ion trap technique

All the six samples made up of *Spirulina* resulted contaminated with desMe-MC-LR, some of these revealed also the presence of other toxins (desMe-MC-RR, MC-LR).

Figure 11. Figure shows the chromatogram of a sample positive for desMeMC-LR (Spir03) compared with that of a recovery at 2000 ng/g spiking level, respective spectra are also reported.

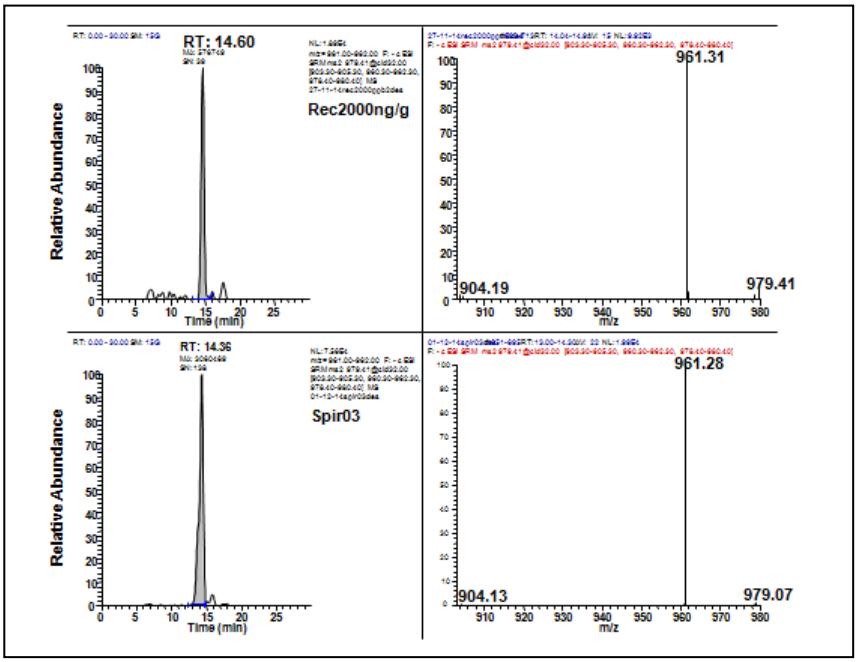
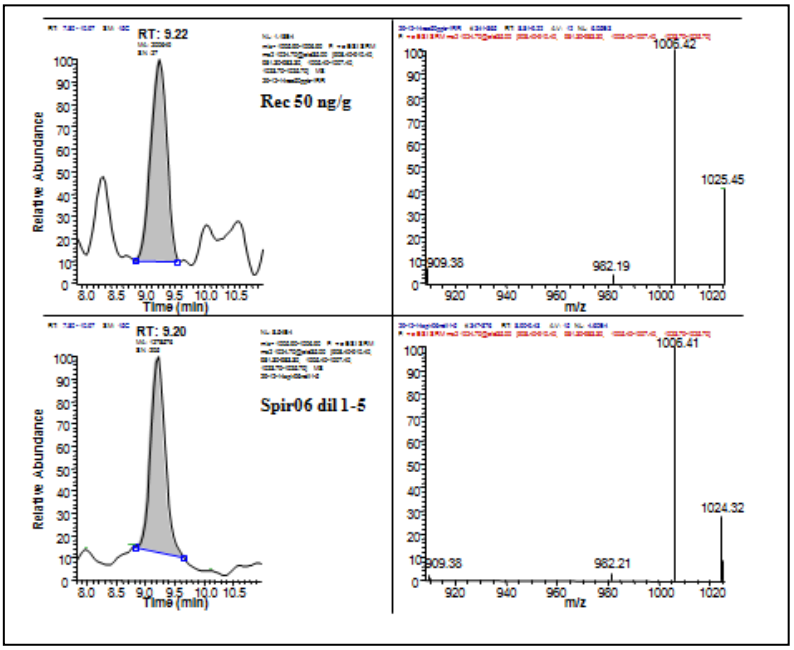
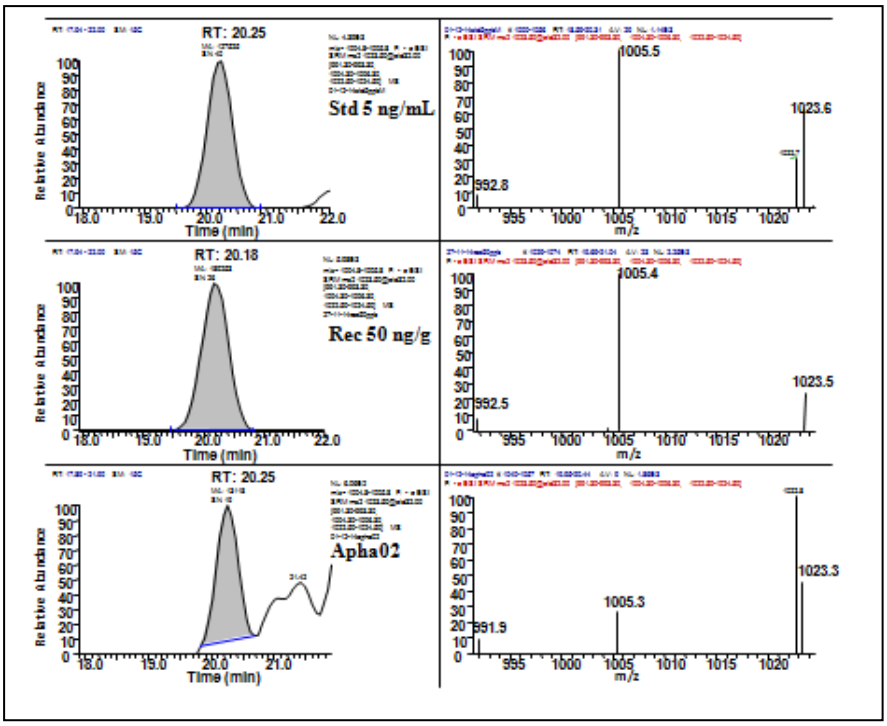


Figure 12. Figure shows the chromatogram of a sample positive for desMeMC-RR (Spir06) compared with that of a recovery at 50 ng/g spiking level, respective spectra are also reported.



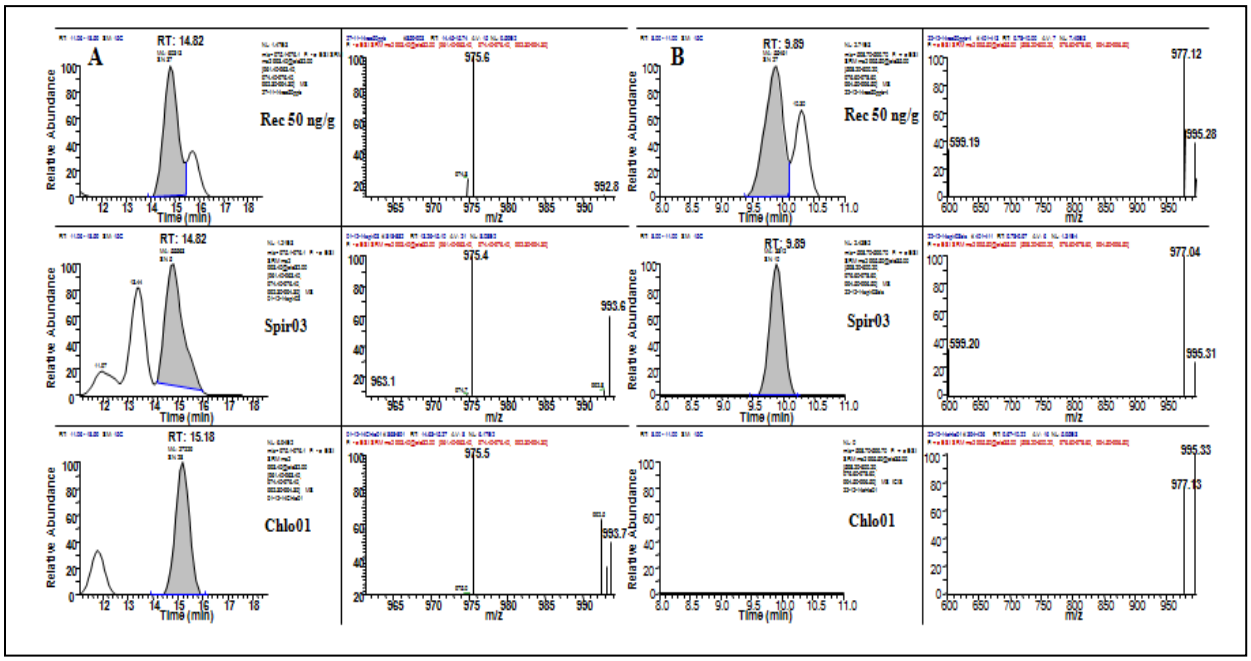
All three samples made up of *Aph. flos-aquae* revealed the presence of MC-LR even if in some cases levels were less than 100 ng/mL and we were not able to confirm the presence of the toxin. One of the samples of *Spirulina* resulted in a high contamination of desMe-MC-RR; traces of MC-LW were found in one sample of *Aph. flos-aquae*, but at a level less than 50 ng/mL, which is our LOQ for this molecule with this technique. Samples resulted in high concentration of the toxins, i.e. Spir02, Spir03, Spir05 and Spir06 were reanalyzed in duplicate.

Figure 13. Figure shows the chromatogram extracted for the SRM identification event of MC-LW of the sample Apha02 compared with that of a recovery at 50 ng/g spiking level and reference standard at 5 ng/g; respective spectra are also reported.



Several samples we analyzed resulted contaminated for MC-LR and we were able to identificate the presence of the toxin with the LC-MS/MS method with the negative ion mode. The substance was not unambigously determinated with the identification point criterion and further analysis were needed in the positive ion mode.

Figure 14. Figure shows the chromatograms extracted for one of the SRM identification event of MC-LR of the sample Spir03 and Chlo01 and compared with that of a recovery at 50 ng/g in the negative (A) and in the positive (B) ion mode; respective spectra are also reported. The presence of the toxin in the sample Chlo01 is not confirmed.



3.13.2 Contaminations of microcystins in dietary supplements-overall results

The analysis of the 11 dietary supplements for the 7 MCCs revealed the presence of at least one of the seven congeners, in all the samples containing *Aphanizomenon* and *Spirulina* cyanobacterial strains. None of the supplements containing only *Chlorella* analyzed resulted in contamination by the 7 MCCs. Overall results are shown in table 24.

Table 24. Table show the results of the analysis of the 11 dietary supplements for the 7 MCCs considered in this study.

Dietary supplement samples	Concentration (ng/g)						
	MC-RR	desMe-MC-RR	MC-LR	desMe-MC-LR	MC-YR	MC-LW	MC-LF
SPIRULINA Spir01	n.r.	n.r.	n.r.	84	n.r.	n.r.	n.r.
SPIRULINA Spir02	n.r.	n.r.	n.r.	2091	n.r.	n.r.	n.r.
SPIRULINA Spir03	n.r.	n.r.	159	1788	n.r.	n.r.	n.r.
SPIRULINA Spir04	n.r.	n.r.	n.r.	208	n.r.	n.r.	n.r.
NUTRASPIRULINA Spir05	n.r.	n.r.	536	3604	n.r.	n.r.	n.r.
ARTHROSPIRA Spir06	n.r.	2825	n.r.	145	n.r.	n.r.	n.r.
ALGA KLAMATH AFA Apha01	n.r.	n.r.	Traces	n.r.	n.r.	n.r.	n.r.
MICROALGHE KLAMATH Apha02	n.r.	n.r.	115	n.r.	n.r.	Traces	n.r.
DIGEVIT Apha03	n.r.	n.r.	Traces	n.r.	n.r.	n.r.	n.r.
CHLORELLA Chlo01	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
VITAMINA B12 & CLORELLA Chlo02	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.

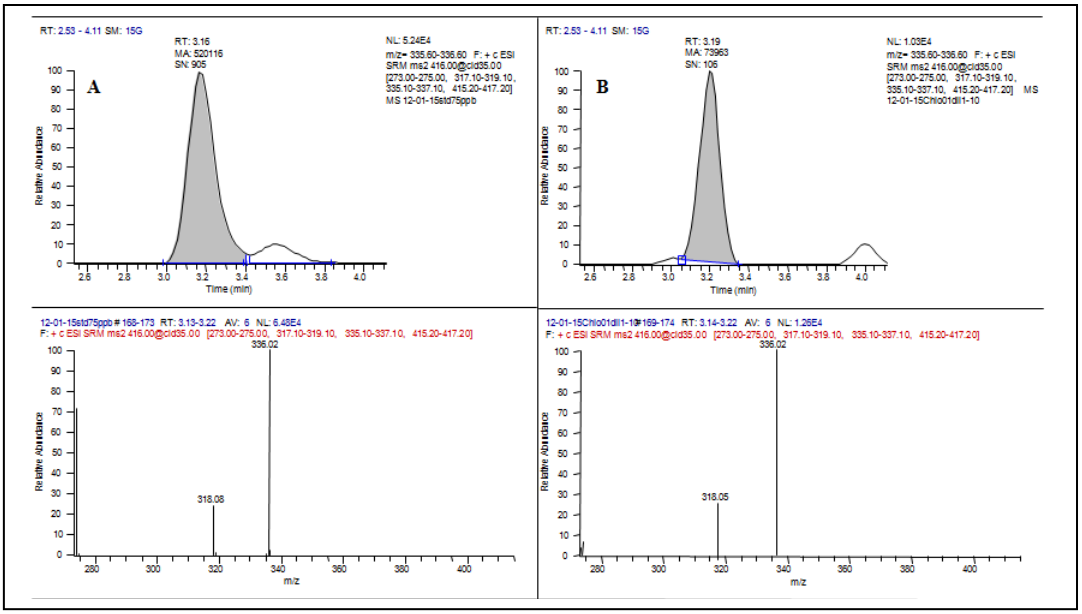
3.13.3 Contaminations of cylindrospermopsin in dietary supplements

The analysis of the 11 dietary supplements for cylindrospermopsin revealed the presence of the toxin in only one of the samples, i.e. CHLORELLA ISWARI (Chlo01). None of the supplements containing *Aphanizomenon* and *Spirulina* cyanobacterial strains analyzed resulted contaminated by cylindrospermopsin. The presence of the toxin was confirmed with the identification point criterion in comparison with an in matrix standard (it scored 3 IP, enough for unambiguous qualitative determination in mass spectrometry in the SRM mode). The contamination level was extimated at a concentration of 120 ng/g with an in-matrix matched standardization.

Table 25. Confirmation of the presence of cylindrospermopsin in the analyzed sample CHLORELLA ISWARI Chlo01 according to the identification point system from the Decision 2002/657/EC.

Decision 2002/657/EC parameters		Cylindrospermopsin diagnostic ions				
Ion relative abundance (% base peak)	Relative abundance tolerance	m/z	Mean relative abundances of the reference standard (% base peak)	Tolerance range calculated on the reference standard (% base peak)	Diagnostic ions from the Chlo01 sample (% base peak)	IP
100 %	± 20 %	336.2	100%	80%-100%	100%	1.5
>20 % to 50 %	± 25%	318.2	27.4%	20.6%-34.2%	25.7%	1.5
>50 %	± 20 %	274.1	71.2%	57.0%-85.4%	6.4%	-

Figure 15. The SRM Chromatograms of an in matrix standard of cylindrospermopsin (A) and (B) CHLORELLA ISWARI (Chlo01) dietary supplements. For each molecule are also reported the MS/MS spectra.



3.13.4 Risk assessment for the consumption of BGA supplements

The World Health Organization (WHO) has fixed a value of total daily intake (TDI) only for MC-LR on the basis of toxicity data. This value for human is 0.04 µg/Kg body weight, applying this value to a 60-kg adult results in 0.04 µg/kg-day × 60 kg = 2.4 µg/day. Assuming a 2 g/day BGA consumption rate (based on product literature and discussions with BGA producers and consumers), 2.4 µg/day ÷ 2 g BGA/day = 1.2 µg/g = 1.0 µg/g. Thus, for adults, a safe level for microcystins in BGA products was determined to be 1 µg/g.
In this work we did a risk evaluation for the consumption of algae supplements according to the dosages indicated by the manufacturer. Results shown in table 26 indicate that, actually, the exposure levels found in 3 samples of the particular brand we have analyzed are in some cases higher than the only toxicity datum TDI assessed for microcystines (referred to MC-LR) by the WHO, also, algae supplements may not be the only font of exposure to MCCs and synergic effects were not considered.

Table 26. Quantity of microcystin ingested per die, according to the dosages of supplement indicated by the manufacturer.

Supplement (commercial name and abbreviation)	Concentration (ng/g)	Indicated dosage	Total quantity of microcystin ingested *per die*
SPIRULINA Spir02	2091	5 × 400 mg pills *per die*	4182 ng
SPIRULINA Spir03	1788	9 × 300 mg pills *per die*	4828 ng
NUTRASPIRULINA Spir05	3604	2 × 525 mg pills *per die*	3784 ng
ARTHROSPIRA Spir06	2825	2000 mg *per die*	5650 ng

3.14 Conclusions 3

Beyond the fact that open water surface cultivation or harvesting of algae products for production of dietary supplements always encompasses the chance that the final products may contain human pathogens, e.g. *Cryptosporidia*, *Campylobacter* and EHEC (Heussner et al, 2012), this analysis clearly demonstrated that a high proportion of the algae dietary supplements available on the Italian market is contaminated with considerable, but varying amounts of MCCs. Thus only prohibition of these products for sale on the national and international markets in combination with strict monitoring by health authorities will protect consumers from serious acute as well as chronic adverse health effects (Vichi et al, 2012).

The MCCs contamination levels found in the analyzed products of the particular brand we have considerate indicate that the exposure for human is in some cases higher than the TDI, and possible synergic effects have not been considered; also, there is no evidence that these products have been monitored for safety respect possible contamination by harmful toxins present as impurities. So our results obtained along with other data from literature demonstrate that there is urgent necessity to implement selective toxicity data and a specific legislation for natural product made up of BGA commercialized in EU Countries. Also, we want to give a particular emphasis to the fact that the most aboundant molecules found were demethylated forms, which eventually are more toxic than their fully methylated counterparts (Ufelmann et al, 2012). As to cylindrospermopsin, to our best knowledge, for the first time an incurred sample of algae supplement was found. The incidence of the toxin and the concentration level compared with the TDI of the toxin indicate that this not constitute a high risk for human health.

3.15 References

Heussner AH, Mazija L, Fastner J, Dietrich DR (2012) Toxin content and cytotoxicity of algal dietary supplements. Toxicology and Applied Pharmacology 265:263-71.

Ufelmann H, Krüger T, Luckas B, Schrenk D (2012) Human and rat hepatocyte toxicity and protein phosphatase 1 and 2A inhibitory activity of naturally occurring desmethyl-microcystins and nodularins. Toxicology 293: 59-67.

Vichi S, Lavorini P, Funari E, Scardala S, Testai E (2012) Contamination by Microcystis and microcystins of blue-green algae food supplements (BGAS) on the Italian market and possible risk for the exposed population. Food Chem Toxicol 50:4493-9.

3.16 Topic 4 – Testing an analytical method for the analysis of aflatoxin M_1 in milk derivative with QuEChERS technique and comparison with the classical approach in IAC

Several studies have already been developed upon the applicability of QuEChERS extraction techniques to mycotoxins in matrix different than vegetables, such as milk, milk derivative, liver and eggs (Aguilera-Luiz et al, 2011, Frenich et al, 2011, O'Mahony et al, 2013, Karaseva et al. 2014). The aim of this study was to test the applicability of QuEChERS extraction techniques to aflatoxin M_1 in milk derivative, with a particular emphasis to ricotta and mozzarella cheese. To do these a series of experiments were performed with the detection in LC-ESI Ion Trap technique to evaluate method performances in comparison with a classical approach in IAC clean-up technique.

3.17 Matherials and methods 4

3.17.1 Reagents

AFM_1 standard was from Biopure (Biopure, Tulln, Austria) in acetonitrile solution at 0.506 µg/mL. Water, for the HPLC mobile phase was produced in a Milli-Q system (Millipore, Bedford, MA, USA). Formic acid and acetic acid (98% grade) were from Carlo Erba (Carlo Erba, Milan, Italy), HPLC-grade methanol was from Merck (Darmstadt, Germany), dichloromethane was from Baker (Mallincrodt Baker, Netherlands), immunoaffinity column were from VICAM (Aflatest, Vicam, Watertown, MA, USA).

3.17.2 HPLC-MS/MS analysis - apparatus

Analysis were performed using a LC/ESI-MS/MS system equipped with a Surveyor LC Pump Plus (Quaternary Pump), a Surveyor Autosampler Plus and a LCQ Advantage ion trap mass spectrometer with an ESI source (ThermoFisher Scientific).
Chromatographic separation was performed by injecting 50 µL of samples onto a 150 × 4.6 mm 5 µm particle Gemini C18 stainless steel column (Phenomenex, Torrance, CA, USA), at 0.35 mL/min, using 0.5% acetic acid in H_2O MilliQ as mobile phase A and 0.5% acetic acid in methanol as mobile phase B. The elution was performed in a gradient mode.
The mass spectrometer was periodically calibrated with solutions of Ultramark, caffeine and Met-Arg-Phe-Ala peptide provided by the manufacturer. During the LC/ESI-MS/MS experiments, mass spectra were acquired in the positive ion mode. The instrument was daily calibrated for the analysis by tuning on AFM_1 pseudo-molecular ion $[M+H]^+$ m/z 329.1.
The LC/ESI-MS/MS analysis were performed by monitoring the signals of the precursor ion → product ion transitions (selected reaction monitoring, SRM mode) from the LC/ESI-MS/MS dataset. The diagnostic ions for MS/MS qualitative analysis are: m/z 259.0, 273.1, 301.0 (base peak), 329.1 (pseudo-molucular peak). The SRM transition selected for quantitative analysis were m/z 329.1 → 301.0 and m/z 329.1 → 273.1.
The data were acquired and processed using the Xcalibur™ software, version 1.3, from ThermoElectron.

3.17.3 Standard solution preparation

A standard solution at 0.100 µg/mL in H_2O/MeOH 1:1 v/v 0.1% acetic acid was prepared by dilution of the mother solution at 0.506 µg/mL and stored in amber-glass vials at 4°C, this solution was stable for 15 days; this was the solution used for the calibration of the MS/MS instrument by flow injection. Another stock standard solution at 10 ng/mL in methanol was prepared by dilution of the mother solution at 0.506 µg/mL and stored in amber-glass vials at 4°C, this solution was stable for 15 days; this solution was used for samples spiking and to prepare the working standard solutions. Calibration working standard solutions in solvent were daily prepared at concentrations of 0.10, 0.20, 0.50, 1.00, 1.50 and 2.00 ng/mL in H_2O/ MeOH 1:1 v/v 0.1% acetic acid, by properly diluting the stock standard solution at 10 ng/mL; the same six calibration working standard solutions were prepared by properly diluting the stock standard solution at 10 ng/mL in a blank extract sample.
Matrix effect of the analyte was estimated by calculating the difference of the linear best-fit slope, obtained from the matrix-matched calibration curve and solvent-based standards calibration curve, divided by the slope of the solvent-based standards calibration curve.

3.17.4 Extraction of blank, spiked and unknown samples with IAC technique

5.0 g of samples (blank, spiked or unknown) of cheeses were put in a 50 mL polipropilene centrifuge tube with dichloromethane (40 mL) and blended for 15 min at high speed. After washing with further dichloromethane (20 mL), the mixture was filtered and the filtrate was then evaporated at 50°C in rotovapor. The residue was dissolved in methanol (1 mL), water (30 mL) and *n*- hexane (20 mL), transferred in a separatory funnel and the aqueous phase

collected. The hexane phase was then washed twice with water (10 mL) and the water phases also collected. The aqueous collections were passed through an immunoaffinity column obtained from VICAM (Aflatest, Vicam, Watertown, MA, USA), according to the instructions of the manufacturer. Subsequently, the column was washed with water and the toxin was then eluted with 2.5 mL acetonitrile/methanol 3:2 v/v, evaporated to dryness under a gentle stream of nitrogen at 50°C and reconstituted to 1000 µL with H_2O/ MeOH 1:1 v/v 0.1% acetic acid.

3.17.5 Extraction of blank, spiked and unknown samples with QuEChERS technique

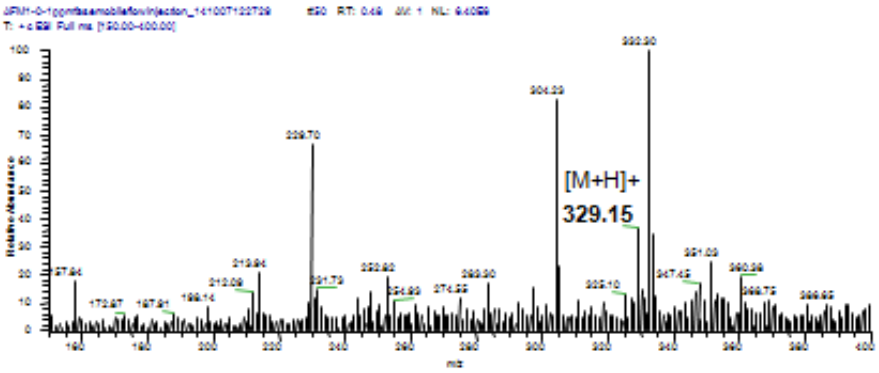
10 ± 0.05 g of samples were weighed into a 50 mL Teflon centrifuge tube. 10 mL MilliQ water and 10 mL 10% formic acid in ACN were added then the tube vortexed for 30 s. The tubes were shaked with an automatic horizontal shaker at maximum speed for 1 h to fully disperse the sample, then a mixture of pre-weighted anhydrous $MgSO_4$, NaCl and citrate buffer (Agilent extraction kit) were added and the tube were shaked immediately and vigorously by hand for 1 min. Tubes were centrifuged at 3500 rpm (2180 rcf) for 5 min; for d-SPE clean-up, 8 mL of the ACN extract (upper layer) were transferred into a 15 mL centrifuge tube containing C18 (0.25 g) and PSA (0.4 g) sorbents, then the tubes were shaked vigorously by hand for 1 min and centrifuged at 3500 rpm for 5 min. 2 mL of the extract were transferred to a glass tube and evaporated to dryness at 40°C under a stream of N_2. 1 mL of mobile phase was added A/B (1:1, v/v) to reconstitute the extract, and then vortexed for 1 min. The extract were filtered using 0.25 µm nylon syringe filter (Chrom Tech Inc., Apple Valley, MN, USA) into autosampler vials.

3.18 Results and discussion 4

3.18.1 Developing ESI-ion trap detection method of AFM_1

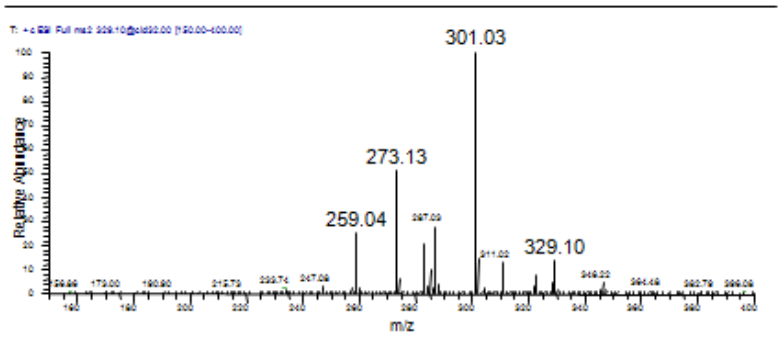
Our goal was to develop a method of analysis of AFM_1 in cheese using up-to-date instruments. We started with optimizing MS/MS conditions with ESI Ion Trap mass spectrometer. We began to study the molecule by injecting a 100 ng/mL dilution in H_2O/MeOH v/v 0.1% acetic acid.
In the positive ion detection mode, the base peak of $[M+H]^+$, with a m/z ratio of 329.1, in the full scan mass spectra served as the precursor ion for optimizing other source parameters, such as the capillary temperature and the capillary voltage, that were finally selected as 300°C and of 4.5 kV respectively.
By tuning on the peak of m/z 329.1, the instrument software automatically set the ion optics and tube lens potentials.

Figure 16. A full scan mode spectrum of the flow injection analysis of AFM_1 100 ng/mL in H_2O/MeOH v/v 0.1% acetic acid



Then, working in the SRM (single reaction monitoring) ion mode, with the precursor ion m/z 329.1, we individuate a fragmentation pattern by optimizing the collision energy at a 32% (instrumental parameter).

Figure 17. The fragmentation pattern at the collision energy of 32%.



The most intense fragment ion generated from the precursor ion was chosen as the quantifier ion (m/z 301.0), whereas the second and third most intense ions were used as qualifier ion for identification (m/z 259.0 and 273.1).

3.18.2 Developing RP-LC separation method of AFM_1

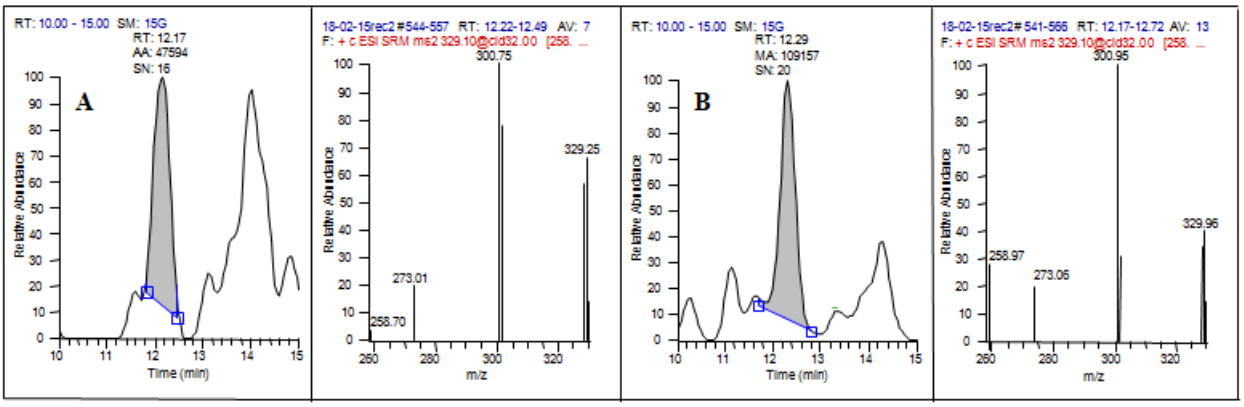
To develop the LC reverse phase elution, a Gemini C18 150 × 4.6 mm 5 µm particle was used; acetonitrile, methanol, water and buffers are commonly used as the mobile phase in LC analysis. We chose 0.5% acetic acid in H_2O MilliQ (mobile phase A) and 0.5% acetic acid in methanol (mobile phase B) because its ionization efficiency and the propriety of methanol to optimize the retention characteristic of the chromatography column we used. Methanol showed to have sufficient solvent strength to fully elute the analyte molecule that seemed to interact very well with the stationary phase, with a symmetric and sharp peak shape. The chromatographic elution was performed in gradient mode as the following programme with a flow rate of 0.35 mL/min: 10 % B held from 0 to 2 minutes,

then from 10 % B to 90% B in 5 minutes, held at 90 % B for 5 minutes; returned to 10 % B over 3 minutes, then held for another 5 minutes.

3.18.3 AFM_1 from milk derivative – a comparison between IAC clean-up and QuEChERS methods

Six artificially spiked milk derivative samples at 0.500 ng/g were extracted and cleaned-up with the two techniques described above. Two samples were of ricotta cheese, two were of buffalo mozzarella cheese and two were of pecorino (Italian sheep cheese). As to method with IAC clean-up technique, we were able to calculate an average recovery over six replicate (63.3%) and a CV% (19.4%), while with the other technique a quite good recovery was obtained for analysis of spiked samples of ricotta cheese (103%, average value over 2 replicates), but quantitative results were not satisfactory for the two other matrices, also in matrix standardization revealed a strong signal suppression and we had to evaluate quantitative results considering another SRM ion transition, i.e. m/z 329.10 → 273.13.

Figure 18. AFM_1 recovery from ricotta cheese with the Quechers technique (A) and IAC technique (B)



3.19 Conclusions 4

QuEChERS clean-up method with PSA/C18 was tested for analysis of AFM_1 in milk derivatives and the test was compared with the classic IAC method clean-up. Results were actually quite satisfactory for the analysis of ricotta cheese, thus revealing that the extraction and clean-up technique is able to recovery the analyte. The limited range of applicability of the method in terms of matrices can be explained most of all with the matrix effects, but, probably, this is a limit of the detection technique employed. We do not exclude the opportunity to develop a QuEChERS method for extraction and clean-up of AFM_1 from milk derivative with more powerful mass spectrometry techniques (such as triple quadrupole or orbitrap) or with a more classic technique, such as HPLC-fluorescence, which is so far the gold standard for AFM_1 detection in the frame of the Official Control (Muscarella et al., 2011).

3.20 References

Aguilera-Luiz MM, Plaza-Bolaños P, Romero-González R, Vidal JL, Frenich AG (2011) Comparison of the efficiency of different extraction methods for the simultaneous determination of mycotoxins and pesticides in milk samples by ultra high-performance liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem 399:2863-75.

Frenich AG, Romero-González R, Gómez-Pérez ML, Vidal JL (2011) Multi-mycotoxin analysis in eggs using a QuEChERS-based extraction procedure and ultra-high-pressure liquid chromatography coupled to triple quadrupole mass spectrometry. J Chromatogr A 1218:4349-56.

Karaseva NM, Amelin VG, Tret'yakov AV (2014) QuEChERS coupled to dispersive liquid-liquid microextraction for the determination of aflatoxins B1 and M1 in dairy foods by HPLC. Journal of analytical chemistry 5:461-466.

Muscarella M, Magro SL, Palermo C, Centonze D (2011) A confirmatory method for aflatoxin M1 determination in milk based on immunoaffinity cleanup and high-performance liquid chromatography with fluorometric detection. Methods Mol Biol 739: 195-202.

O'Mahony J, Clarke L, Whelan M, O'Kennedy R, Lehotay SJ, Danaher M (2013) The use of ultra-high pressure liquid chromatography with tandem mass spectrometric detection in the analysis of agrochemical residues and mycotoxins in food - challenges and applications. J Chromatogr A 1292:83-95.

3.21 Glossary

AFM_1	Aflatoxin M_1
ACN	Acetonitrile
BGA	Blue Green Algae
CYN	Cylindrospermopsin
DAD	Diode Array Detector
EC	European Community
EU	European Union
EFSA	European Food safety Association
ELISA	Enzyme linked immunosorbent assay
EHEC	Enterohemorrhagic *Escherichia coli*
ESI	Electrospray Ionization
FAO	Food and Agriculture Organization
FLD	Fluorescence Detector
GTS	Glucose Transport System
HPLC	High Performance Liquid Chromatography
IAC	Immuno-Affinity Chromatography
IARC	International Agency for Research on Cancer
LCT_{50}	Lethal Concentration Time 50 %
LD_{50}	Lethal dose 50 %
MALDI	Matrix Assisted Laser Desorption Ionization
MCC	Microcystin
MS	Mass Spectrometry
NOEL	Not Observed Effect Level
PCR	Polymerase chain reaction
PDA	Photo Diode Array
PSA	Primary/secondary ammine
PMTDI	Provisional Maximum Total Daily Intake
Q-TOF	Quadrupole-Time of Flight
Q-Trap	Quadrupole-Trap
QuEChERS	Quick Easy Cheap Effective Rugged Safe
SPE	Solid phase extraction
TDI	Total Daily Intake
WHO	World Health Organization

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APPENDIX 1: scientific productions

Papers

Gallo, P., **Fabbrocino, S.** & Serpe L., 2012. Determination of levamisole in feeds by liquid chromatography coupled to electrospray mass spectrometry on an ion trap. Rapid Comm. in Mass Spectr. 26: 733-739.

Ferranti, P., **Fabbrocino, S**. Chiaravalle, E., Bruno, M., Basile, A., Serpe, L., & Gallo, P. 2013. Profiling Microcystins contamination in a water reservoir by MALDI-ToF and liquid chromatography coupled to Q/TOF tandem mass spectrometry. Food Res. Int. 54: 1321-1330.

Conference proceedings

Gallo, P., Vinci F., **Fabbrocino S**., Fiori M. & Serpe L. 2011, Validazione di metodi multi-residuo per l'analisi degli AINS in plasma e attività di supporto alle indagini giudiziarie. "II Convegno Nazionale Ricerca in Sanità Pubblica Veterinaria". Roma.

Gallo, P., **Fabbrocino, S**. & Serpe, L. 2012, Determination of levamisole in feeds by liquid chromatography coupled to electrospray mass spectrometry on an ion trap. "Euroresidue VII – 2012", Egmond Aan Zee -The Netherland.

Gallo, P., **Fabbrocino, S.,** Serpe, L. & Guarino, A. 2012, Determinazione di microcistine in integratori a base di alghe mediante LC/ESI- MS/MS in trappola ionica. "XIV Congresso Nazionale S.I.Di.L.V". Sorrento (NA).

Gallo P., Urbani V., Guadagnuolo G., **Fabbrocino S.**, Serpe L., Fiori M., Civitareale C., Stacchini P. & Muscarella M. 2014, Exploring occurrence of coccidiostats and ionophores below tolerable cross contamination levels by HPLC-DAD and Ion Trap LC/ESI-MS/MS methods. "7th VDRA Ghent Belgium". 2-5 June 2014.