A Review of Methods for the Simultaneous Detection of Illegal Ingredients in Food Supplements

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Summary

Food supplements are at risk from contamination with illegal ingredients on a global scale. To date, the official food control laboratory system in the UK does not appear to have been particularly active in the analytical control of illegal ingredients in food supplements. From a survey of notifications (2009 to 2016) to the European Union rapid alert system for food and feed (RASFF) food supplements are shown to be adulterated with a complex range of compounds and substances. These include permitted food additives in excess of their limits, contaminants, unauthorised novel food ingredients, unauthorised nutritionally-related compounds, excess vitamins, controlled drugs, and one instance of the poison strychnine.

However, arguably, the most important factors that jeopardise the safety of food supplements are their adulteration with synthetic pharmaceutical drugs. In order to assist official control and trade analysts to regulate the safety of food supplements with regard to the presence of illicit pharmacologically active ingredients or contaminants the RASFF database, 2009 to 2016, was surveyed for such compounds. The most frequently notified were sildenafil and its analogues, sibutramine and derivatives, 1,3-dimethylamylamine (DMAA), yohimbine and tadalafil. Their toxicology and methods for their detection and determination have been reviewed in this paper. Method details are tabulated with references and general conditions have been suggested for a first choice liquid chromatography-mass spectrometry (LC-MS) screening method for the compounds sildenafil, tadalafil, vardenafil and yohimbine. If high field NMR is available this would appear to be an excellent first-line method of control for herbal food supplements.

It would be helpful that when the suggested method(s) are assessed and validated in Association of Public Analyst member laboratories against a matrix of food supplements incurred or spiked with the target illegal ingredients that the data be reported herein.

Introduction

Food supplements are at risk from contamination with illegal ingredients on a global scale. To date, the official food control laboratory system in the UK does not appear to have been particularly active in the analytical control of illegal ingredients in food supplements. One reason perhaps is the lack of a list of prioritised illegal ingredients, and convenient analytical method(s) to screen for them. Hence these problems have been examined to assess which illegal ingredients should be prioritised and the potentially useful methods of analysis have been reviewed in order to suggest a "*method of first choice*" that can be used to screen for an optimum range of the prioritised illegal ingredients.

Food Supplements

Food supplements are concentrated sources of substances with nutritional or physiological effect and are intended to supplement the normal diet[1,2]. They include a spectrum of dietary ingredients including, but not limited to, herbs, botanicals, vitamins, minerals and enzymes (or extracts) from organs or glands[3-5]. Responsibility for legislation on food supplements in England was transferred from the Food Standards Agency (FSA) to the Department Health (DH) on 1 October 2010. In Northern Ireland it remains with FSA whilst in Wales the responsibility lies with the Welsh Assembly and in Scotland with Food Standards Scotland (FSS).

A food supplement is defined as "*any food the purpose of which is to supplement the normal diet"* and which is *"a concentrated source of a vitamin or mineral or other substance with a nutritional or physiological effect, alone or in combination, and sold in dose form"*. In the UK the Food Supplements Regulations 2003 permit the sale of food supplements provided they contain approved vitamins and minerals which are as listed respectively in Annexes I and II of the overarching EC Directive 2002/46[6]. However, to date in the UK the official laboratory control system has not, unlike their counterparts in Italy[7-9], been particularly active in the analysis of illegal ingredients in food supplements. This appears to be due to the lack of a list of prioritised illegal ingredients and of convenient methods for their detection/determination. Food labelling law, which also covers food supplements, prohibits any claim that a food or food supplement can prevent, treat or cure any disease and is dealt with by EC Regulation 1924/2006[10] in the light of updates to Directive 2002/46[11-14].

Since food supplements are now commonly used by people as part of their personal healthcare regimen there has been a tremendous growth in the supplies and sales of these products. Food supplements can be easily purchased from pharmacies, health food stores and from internet sites with direct delivery thus lacking any border control or surveillance. These products are promoted worldwide, often with unverified benefit claims and without adequate information of potential hazards[3,15,16]. Many people consume large quantities of food supplements without knowing the potential interactions with other supplements or drugs that they may be taking in parallel. Food supplements are regulated as foodstuffs and not with the same pre-sales rigour as medicines[10-14]. Hence, the safety of food supplement consumption is often questionable.

Safety of Food Supplements

The most important factors that jeopardise the safety of food supplements are their adulteration with synthetic pharmaceutical drugs and from inappropriate labelling. The presence of undeclared substances in food supplements has become a common problem leading to serious health risks such as acute or chronic toxicity, severe side effects and drug interactions. Some manufacturers have been found attempting to improve the efficacy of their products[16,17] to deal with common problems such as those of diabetes/obesity[18-22], pain[23] and erectile dysfunction[7,24-29] by the intentional addition of undeclared active ingredients. Hence, consumers are deceived into thinking that they are getting the claimed health benefit from an unadulterated product and may also be unaware of the potentially serious health risks that they are exposed to when consuming such products.

Adulteration can also occur accidentally during the manufacture or distribution of food supplements via contaminated ingredients in the supply chain that manufacturers of the finished product may then unintentionally make use of. Poor quality control measures during production can also lead to the contamination of supplements. There are several reports on toxicological problems owing to exposure to food supplements, highlighting the challenges and the need to regulate such products[29-33].

Survey of Illegal Ingredients in Food Supplements

The European Union operates a rapid alert system for food and feed (RASFF) to ensure food safety (http://ec.europa.eu/food/safety/rasff/index_en.htm). Adverse analytical findings are logged into this database which allows the Member States to inform one another of the risks to health from food and animal feeds. Similar records are kept by FDA (http://www.fda.gov/Safety/Recalls/EnforcementReports/default.htm). A search of undeclared and unauthorised substances frequently reported in food supplements by RASFF each year from 2009-2016 was carried out. Compounds of potential pharmacological activity were recorded and are listed in Table 1. The presence was observed, (but not tabulated herein), of excess food additives, contaminants (including microbiological agents, heavy metals, pesticide residues and mycotoxins), unauthorised novel food ingredients, unauthorised nutritionally related compounds (for example, bis (picolinato) oxo vanadium, betaine, metal amino acid chelates etc) and excess vitamins. There were also some instances of the controlled drug tetrahydrocannabinol (THC) and one instance of the poison strychnine.

From the data in Table 1 it can be seen that the most frequently found undeclared pharmacological ingredients in food supplements in the years 2009 to 2016 were sildenafil and its analogues, sibutramine and derivatives, 1,3-dimethylamylamine (DMAA), yohimbine, and tadalafil. Other interesting adulterants exhibiting frequent notifications include the biogenic amine synephrine and derivatives, and phenolphthalein both of which are of toxicological concern and implicated in attempted weight loss. A pilot study of FDA data (2009-2013) presented in Table 2 confirms that the above priority list is likely to reflect the US experience. In order to try to avoid detection unapproved active analogues of most of these drugs have also been frequently reported as adulterants[7-9,17]. Note in this context

DMMA is not to be confused with the psychoactive drug 3,4-Dimethoxy-N-methylamphetamine which is also known by the acronym DMMA.

Table 1 - List of Undeclared and Unauthorised Pharmacological Substances frequently reported in Food Supplements by the RASFF

Substance	2009	2010	2011	2012	2013	2014	2015	2016*	Total
Sildenafil (including analogues)	3	13	7	10	10	15	5	5	68
Sibutramine and derivatives	6	10	14	21	4	4	0	4	63
DMAA	0	0	0	36	5	7	3	7	58
Synephrine, phenethylamine and derivatives	1	1	1	1	7	10	3	13	37
Yohimbine	1	4	1	1	6	8	2	7	30
Tadalafil	3	3	11	5	2	3	2	0	29
Phenolphthalein	0	2	2	6	7	0	0	3	20
Vinpocetine	6	1	7	0	0	1	0	0	15
Melatonin	3	3	0	1	1	0	0	0	8
Androstenedione	0	2	0	0	0	3	1	0	6
Progesterone	1	1	0	0	0	2	1	0	5
Dehydroepiandrosterone (DHEA)	1	2	0	0	0	0	0	1	4
Octopamine or derivatives	0	0	2	1	0	0	0	1	4
Dithiodesmethylcarbodenafil	0	0	2	1	0	0	0	0	3
Idebenone	2	0	1	0	0	0	0	0	3
Nor-acetildenafil	1	1	1	0	0	0	0	0	3
Vardenafil, Pseudovardenafil	0	0	2	0	0	1	0	0	3
Vincamine	0	0	3	0	0	0	0	0	3
Vinburnine	0	0	3	0	0	0	0	0	3
Diindolylmethane (DIM)	0	0	0	0	0	1	0	1	2
Evodiamine	0	0	1	0	0	1	0	0	2
5-hydroxytryptophan (5-HTP)	0	0	0	1	0	0	1	0	2
Stanozolol	1	0	0	0	0	0	1	0	2
Acetildenafil	0	1	0	0	0	0	0	0	1
2,4-dinitrophenol (DNP)	0	0	0	0	0	0	0	1	1
7, 17 dimethyltestosterone	0	1	0	0	0	0	0	0	1
Indole-3-carbinol	0	0	0	0	0	1	0	0	1
Methylsulphonylmethane	0	0	0	0	0	0	0	1	1
Nimesulide	1	0	0	0	0	0	0	0	1

*To 31/08/2016,
Search criteria 'blank', Category – dietetic foods, food supplements and fortified foods

Table 2 - List of Undeclared and Unauthorised Substances frequently reported in Food Supplements by the FDA, 2009 – 2013

	2009*	2010	2011	2012	2013	Total
Sibutramine	0	1	7	2	6	16
Sulfoaildenafil	0	6	6	1	0	13
Sildenafil	0	1	2	4	3	10
Tadalafil	0	0	2	5	2	9
Hydroxythiohomosildenafil	0	2	0	2	1	5
Dimethylsildenafil	0	0	2	0	1	3
Aminotadalafil,	0	1	0	0	1	2
ephedrine alkaloids	0	0	1	1	0	2
Sulfohydroxyhomosildenafil	0	0	0	0	1	1
Desmethyl Carbodenafil	0	0	0	0	1	1
N-di-Desmethylsibutramine.	0	0	0	0	1	1
N-Desmethyl Sibutramine	0	1	0	0	0	1
DMAA	0	0	0	0	1	1
Unspecified analogue of sildenafil	0	0	0	0	2	2
Superdrol, Madol, Tren, Androstenedione, and/or Turinabol	0	2	1	0	0	3
Phenolphthalein	0	0	0	0	2	2

* from 21/04/2009
http://www.fda.gov/Safety/Recalls/default.htm
Search criteria 'blank', Tab-All recalls

Aspects of the Toxicology of the Prioritised Illegal Ingredients in Food Supplements

Tadalafil and sulfoaildenafil are analogues of sildenafil and belong to the group of phosphodiesterase-5 inhibitors (PDE-5i). These drugs are prescribed for the treatment of erectile dysfunction in men. It has been reported that PDE-5i's can interact with other drugs containing nitrates (eg nitroglycerin) to lower blood pressure drastically. People suffering from conditions like diabetes, hyperlipidemia and hypertension are frequently prescribed nitrate containing medicines. Furthermore, erectile dysfunction is often associated with these conditions[34]. As PDE-5i are contraindicated in such patients they are often inclined to take food supplements, mainly herbals, for their treatment of erectile dysfunction. If these supplements are adulterated with PDE-5i's it can lead to serious health consequences.

Yohimbine has been reported to have aphrodisiac-like effects. However, it is not sufficiently safe to be freely available for uncontrolled use. Daily doses of 15mg have been reported to cause bronchospasm and a lupus-like syndrome[35]. It is known to increase blood pressure and induce anxiety. Toxicity of yohimbine can be enhanced when taken concomitantly with drugs like phenothiazines[35]. Furthermore, the possibility of yohimbine causing fat loss in elite athletes has been reported[36].

Sibutramine is approved for the medically-controlled treatment of obesity. However, studies have indicated that sibutramine use might be associated with adverse effects such as panic attacks, memory impairments and psychotic episodes[18].

DMAA (1,3-dimethylamylamine, methylhexanamine or geranium extract) is a sympathomimetic compound patented in the 1940s for use as a nasal decongestant. It has been reported to be a widely-used ingredient in sport food supplements as a recreational stimulant. There is now some speculation of DMAA being sourced naturally[37]. In 2012, the FDA issued warning letters to companies that resulted in products containing DMAA being taken off the market or reformulated to remove this substance. In 2013, as a result of FDA action, a food supplement firm destroyed DMAA-containing products which are estimated to have been worth more than $8 million at the retail level, (http://www.fda.gov/Food/DietarySupplements/ProductsIngredients/ucm346576.htm).

Thus, to ensure the quality of food supplements and protect consumer's health, it is important to have adequate methods for the analysis of these illegal and potentially toxic compounds in food supplements. There are numerous methods reported for the analysis of these substances in a variety of food supplements with the most commonly sought life style enhancements, such as reduction in obesity and improving sexual performance. Although high performance liquid chromatography (HPLC) has been the most popular approach, high field ^{1}H NMR spectroscopy has been used with considerable success for the detection, identification and quantification of adulterants in herbal dietary supplements marketed for improving sexual performance[27] and for weight loss[21].

Analytical Methods for the Prioritised Illegal Ingredients in Food Supplements

Numerous HPLC methods have been reported for the detection of the above-mentioned prioritised illegal ingredients in food supplements. Most commonly, bonded octadecyl silane (C-18) has been used as the stationary phase, with a combination of an acidic aqueous solution and acetonitrile for the mobile phase, as shown in summary in Table 3.

Table 3 - Summary of Liquid Chromatographic Methods for the Detection of the Top Six Adulterants in Food Supplements

Analytes of Interest	Sample Extractant	Column	Mobile Phase		Detector	Reference
			Solvent A	**Solvent B**		
Sibutramine	methanol	C18	0.1 % formic acid in water	0.1 % formic acid in acetonitrile	MS	[18]
	methanol	C18	0.1% sodium-1-hexane-sulfonate in 0.1% phosphoric acid	acetonitrile	PDA	[18]
	methanol	C18	0.1% formic acid	acetonitrile	MS	[40]
	Acetonitrile:water, 50:50	ACE phenyl	0.19M formic acid	acetonitrile	Fluorescence	[41]
	methanol	C8	0.2% formic acid and 20mM ammonium acetate	acetonitrile	MS	[42]
Sibutamine and other slimming agents	methanol	C18	water with 0.025 M ammonium acetate	acetonitrile	UV	[22]
	Methanol:water, 70:30	C18	water with	acetonitrile with	MS	[20]

Analytes of Interest	Sample Extractant	Column	Mobile Phase		Detector	Reference
			Solvent A	**Solvent B**		
			0.1% formic acid, 5% acetonitrile	5% water		
	methanol	C18	water with 5mM ammonium formate	acetonitrile with 0.1% formic acid	MS	[8, 19]
Sildenafil vardensafil and tadalafil	liquid samples diluted with methanol	C18	water with 20 mmol/l ammonium acetate and 0.2% formic acid (v/v)	acetonitrile	MS	[24]
	acetonitrile	C18	water with 0.01M ammonium formate	acetonitrile	MS and DAD	[43]
	acetonitrile:water, 50:50; methanol	C18	water with 0.1% formic acid	acetonitrile	MS	[9, 34, 38]
Codeine and other analgesics	methanol with 1% formic acid	C18	water with 0.025 M ammonium acetate	methanol	UV	[23]
DMAA, yohimbine, sildenafil, tadalafil, sibutramine	acetonitrile with 2% formic acid	Acquity UPLC HSS T3 reversed phase	water with 5 mM ammonium formate with 0.1% formic	methanol containing 5 mM ammonium formate with 0.1% formic acid.	HRMS	[39]

Analytes of Interest	Sample Extractant	Column	Mobile Phase		Detector	Reference
			Solvent A	**Solvent B**		
			acid.			
DMAA	0.5M HCl	C18	water	acetonitrile with 0.1% formic acid	MS	[44]
	0.5M HCl	C18	water with 1% formic acid	acetonitrile	MS	[45]
Yohimbine, sildenafil, tadalafil	methanol	C18	0.1% acetic acid in water	acetonitrile	MS	[25]
Sildenafil, vardenafil, tadalafil and analogues	methanol/water 70:30	C18	water with 2mM ammonium formate and 0.2% formic acid	0.2% formic acid in 95% acetonitrile	MS	[33]

Suggested Screening First Choice Method

Based on the data above a general method of first choice is that of Zhang *et alia*[25] for yohimbine, sildenafil, vardenafil and tadalafil as follows:

MS Conditions

Triple quadrupole mass spectrometer in ESI positive ion mode
Suggested MRM transitions (Zhang *et alia* [25]):

Compound	**Suggested Transitions *m/z* (a) quantitative**
Yohimbine	355.2 → 144.1 (a)
	355.2 → 212.4
Sildenafil	475.3 → 100.1(a)
	475.3 → 58.0
Vardenafil	489.5 → 151.1 (a)
	489.5 → 312.1
Tadalafil	390.4 → 268.3 (a)
	390.4 → 135.2

Chromatographic Conditions

Column:	C18 (150 mm x 4.6 mm)	
Flow rate:	0.5 mL/min	
Column temperature:	30^{o}C	
Injection volume:	10µl	
Mobile phase a:	0.1% acetic acid in water	
Mobile phase b:	acetonitrile	
Gradient elution programme:	0-3 min:	20% B
	3-8 min:	20-95% B
	8-13 min:	95% B;
	13-1301 min:	95-20% B
	1301-20 min:	20% B.

This method can be extended for the analysis of the sulfoaildenafil by a change of eluant A. The use of a split flow split can be employed to simultaneously carry out photodiode array (PDA) analysis.

In passing it may be helpful to note that Roman *et alia* have published an LC-UV method for synephrine in bitter orange raw materials, extracts, and dietary supplements[46].

Summary and Conclusions

From the data considered herein food supplements are shown to be quite often adulterated with a complex range of compounds and substances. These include permitted food additives in excess of their limits, contaminants, unauthorised novel food ingredients, unauthorised nutritionally related compounds, excess vitamins, controlled drugs and one instance of the poison strychnine. For most of these adulterants official control laboratories have validated methods in place. However, in order to assist official control and trade analysts to regulate the safety of food supplements with regard to the presence of illicit pharmacologically active ingredients or contaminants:

- The RASFF database (2009 to 2016) has been surveyed to reveal the most frequently found undeclared pharmacologically active ingredients in food supplements in these years. These were sildenafil and its analogues, sibutramine and derivatives, 1,3 dimethylamylamine (DMAA), yohimbine, and tadalafil

- Methods have been reviewed for the detection and determination of the above most common adulterants

- The general conditions have been suggested for a first choice HPLC-MS screening method for the above compounds

- If high field NMR is available this would appear to be an excellent first-line method of control for herbal food supplements

It would be helpful that when the suggested method(s) are assessed and validated in APA member's laboratories against a matrix of food supplements incurred or spiked with the target illegal ingredients that the data be reported herein.

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