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Development and optimization of innovative analytical methods for the determination of phytoestrogens in soy-food by chromatographic techniques coupled to mass spectrometry

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Chi ha un perché abbastanza forte,

può superare qualsiasi come.

F. Nietzsche

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Abstract

The present PhD thesis deals with the development and optimization of different analytical methods, which combines powerful instrumental techniques and innovative pre-treatment methods to obtain reliable quantitation. Both gas chromatography and liquid chromatography coupled to mass spectrometry (GC-MS and LC-MS) were applied for the determination of specific analytes in several matrices.

In particular, the main focus was on the quantitation of phytoestrogens in soy-based food matrices. These compounds are interesting because of their endocrine disrupting potential, and their accurate determination is necessary in "non-traditional" foods which are new on the market, especially in Italy. The instrumental analysis of these compounds was optimized by accurate studies of the different parameters involved in GC-MS and LC-MS analyses, in order to obtain maximum sensitivity, specificity and reproducibility. Optimization of the sample preparation was another fundamental aspect of the work and was carried out by exploiting the chemometric approach of experimental design.

Alongside the main topic of phytoestrogens quantitation in food matrices, an environmental study was performed and dealt with the development of an innovative extraction method for the determination of a class of contaminants (polycyclic aromatic hydrocarbons) in sea water.

A range of preparative and instrumental techniques were studied, compared and applied to environmental and food samples; analytical performances were assessed for all the developed methods and compared with the available literature.

Chapter 1

Introduction

1.1 What are phytoestrogens?

Phytoestrogens are a wide group of natural non-steroidal compounds, belonging to the general family of polyphenols, widespread in the plant kingdom and considered as xenoestrogens. In plants, they are found as secondary metabolites, i.e. chemicals which are not normally essential to life, but are produced in response to specific stimuli. In fact, their synthesis generally occurs when the organism is subjected to environmental stresses, such as pathogens infection and water or nutriments scarcity [1,2]. In particular, many functions have been attributed to phytoestrogens, such as role as pigment precursors, lignification agents and excretion products as well as plant-microbe symbiotic functions. Several authors also suggested an implication in reproductive processes and protective effects in pest infections [3]. In mammals, they mainly exhibit estrogenic activity; they are able to interact with the endocrine system through a wide range of mechanisms, being considered as endocrine disruptor chemicals (EDCs). For the complexity of phytoestrogens action mechanisms, the attribution of positive or negative effects on human health is still controversial.

Phytoestrogens are divided in different classes on the basis of the chemical structure and biosynthesis patterns; main groups are chalcones, flavonoids, lignans and stilbenoids [4]. Among them, the most known are flavonoids and lignans; their occurrence in plants and effects on animals and humans have been extensively studied. Flavonoids are in turn categorized in subclasses, which include, among others, isoflavones and coumestans, known as the ones having the strongest estrogenic properties.

The group of isoflavones is probably the most deeply investigated. Isoflavones' structure is made of a central heterocycle and two external substituted phenolic rings (Fig. 1). The most studied isoflavones are daidzein and genistein, found in nature as aglycones and glycosides (daidzin and genistin, respectively) and their methoxylated derivatives formononetin and biochanin A. These compounds have shown significant estrogenic potential. The main sources of isoflavones are plants belonging to the Leguminosae family [5], such as common bean (Phaseolus vulgaris), chickpea (Cicer arietinum) and, above all, soybean (Glycine max). Other isoflavone-rich species are red clover (Trifolium pratense), alfalfa (Medicago sativa) and licorice (Glycyrrhiza glabra)[4].

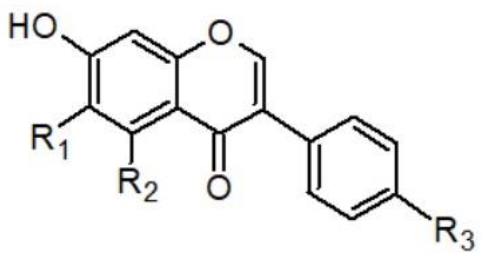


Figure 1: general structure of isoflavones; R_1, R_2 and R_3 indicates substituents.

Coumestans are chemicals biosynthetically derived from isoflavones; the basic structure is characterized by 4 rings, with two central heterocycles of six and five carbon atoms and two external phenolic groups (Fig. 2). Coumestrol is the main representative of this group, known for being one of the most potent phytoestrogens. The richest source of this compound is alfalfa (Medicago sativa) but high concentrations are also found in clover, soybeans and spinaches [4].

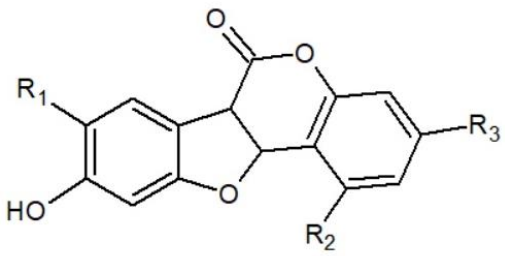


Figure 2: general structure of coumestans; R_1, R_2 and R_3 indicates substituents.

Although phytoestrogens are natural plants components, several other matrices may contain them; in fact, because of the feeding and subsequent metabolism of these substances by breeding animals,

phytoestrogens can be found in products of animal origin, such as meat, eggs or milk. The intake of phytoestrogens by cattle can derive both from grazed plants or soy-based feedings, which are more and more diffused, thus contributing to the increase of concentration in these matrices [6]. However, soy surely represents the most important dietary source of phytoestrogens, especially isoflavones.

Even though plant and food matrices are the main phytoestrogens sources, we have to keep in mind that non-negligible concentrations can be found in the environmental compartment, where they are considered emerging contaminants. In fact, these compounds can be released in the environment after animal and human excretion, but also because of water discharges from plants manufacturing soy and other vegetables.

As far as the human metabolism is concerned, after ingestion, phytoestrogens are involved in complex metabolic pathways. Generally, the most abundant species in plants are the glycosylated forms, because of the higher water solubility; these biologically inactive glycosides are readily hydrolysed in the intestine to the aglycones, which are easily adsorbed. Then, they reach the plasma peak concentration in 4-7 hours and are excreted after 7-9 hours [7]. Daidzein can also be metabolized in equol, an isoflavandiol, by the gut microflora, but only the 30-50% of humans are able to make this conversion [8]. This source of variability could account for the inter-individual differences reported as far as healthy or detrimental effects of phytoestrogens are concerned.

Inside the class of phytoestrogens, during this work of thesis, the focus was on the four isoflavones Daidzein, Genistein, Formononetin and Biochanin A, and the coumestan Coumestrol, which are shown in Fig. 3. The main chemico-physical properties of these analytes are summarized in Table 1 [9,10].

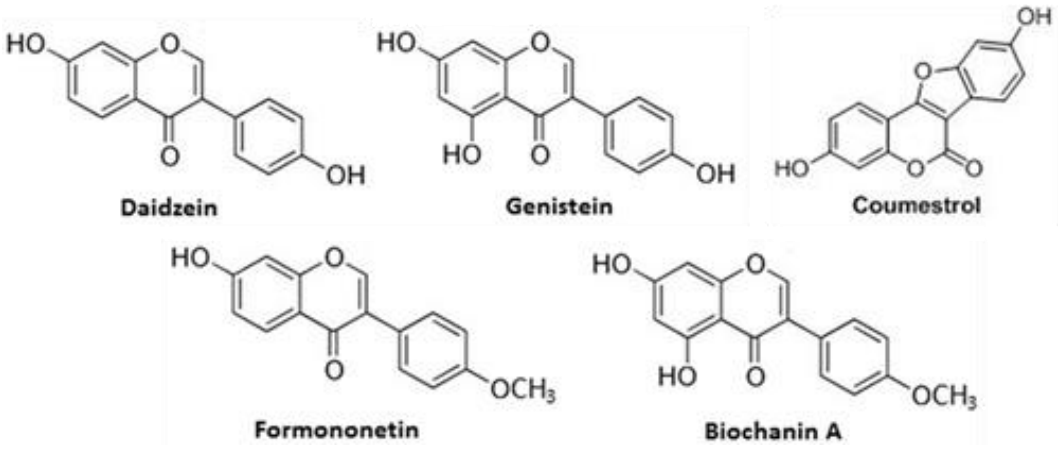


Figure 3: structures of the five phytoestrogens chosen for the work.

Table 1: chemico-physical properties of the investigated analytes.

	Molecular weight (g/mol)	**Log kow (extimated)**	**pka (most acidic moiety)**	**Solubility in water**	**Melting point (°C, extimated)**	**Boiling point (°C, extimated)**
Daidzein (DAID)	254.2375	2.5	6.48	insoluble	315	317
Genistein (GEN)	270.2369	2.84	7.63	insoluble	297-298	333
Coumestrol (COUM)	268.22102	2.8	7.11	0.28 g/L	385	not available
Formononetin (FORM)	268.26408	2.8	6.48	0.04 g/L	256-258	not available
Biochanin A (BIOCH)	284.26348	3	6.55	0.058 g/L	not available	not available

1.2 Effects on animals and humans

As already mentioned, phytoestrogens are considered endocrine disruptors, since their activity resembles the definition of this kind of substances. In fact, EDCs are defined as "exogenous agents that interfere with synthesis, secretion, transport, metabolism, binding action or elimination of natural hormones in the body that are responsible for homeostasis, reproduction and development and behavior" [7]. EDCs are contaminants of emerging concern, since exposure to them can cause adverse effects on several aspects of reproduction, for instance sexual development, timing of puberty, fertility and pregnancy. The endocrine disrupting potential of phytoestrogens was recognized for the first time in the 1940s, when a paper was published on the reproductive problems of Australian sheep grazing clover [11]. The high intake of formononetin, because of the ingestion of large amounts of red clover, caused infertility and was called the "clover disease". In the following years, other studies gave similar indications on the endocrine disrupting capacity of phytoestrogens.

The interaction of phytoestrogens with the endocrine system is mainly due to their capacity to bind to the estrogens receptors (ER), both α and β. Some phytoestrogens possess a chemical structure similar to that of estradiol; all of them present at least one phenolic group in common with estradiol and the most active ones possess a second hydroxyl group on the opposite side of the molecule, mimicking the angle and distance of this two groups in the estradiol molecule [3], as shown in Fig.4.

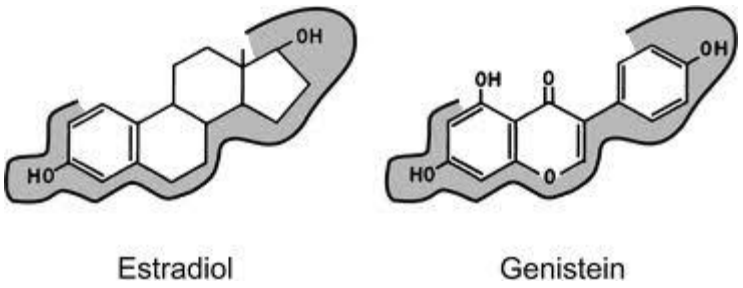


Figure 4: structural similarity of the isoflavone Genistein with respect to the natural estrogen Estradiol (from http://aiocm.org/wp-content/uploads/2012/03/images.jpeg).

Phytoestrogens may act as estrogen agonists or antagonists, or none of them, depending on the particular chemical structure, on the relative abundance of α or β receptors (tissue specific) and on the concentration of natural estrogens. As agonists, they can bind to ERs activating them and producing an estrogenic effect; as antagonists they can block or alter the binding of natural hormones, thus causing an anti-estrogenic effect. For this reason, they are defined as selective estrogen receptor modulators (SERM) and their action is normally organ dependent. Independently from the affinity to ERs, they can also be involved in inhibition or activation of enzymes connected with endocrine functions [4,12], thus inducing an indirect estrogenic effect.

During the years, many macroscopic effects have been ascribed to phytoestrogens, both positive or detrimental, by several experimental or epidemiological studies. The apparent discrepancies found in the literature are due to the high complexity of the action mechanisms, which often depend on several factors, such as exposition to other substances, interspecies differences and individual characteristics, making it difficult to obtain clear and definitive conclusions.

For a long time, thanks to *in vitro* studies and *in vivo* animal experiments, as well as epidemiological studies, positive effects on human health have been attributed to phytoestrogens. Phytoestrogens are known to alleviate menopausal symptoms; their estrogenic effect can reduce intensity and frequency of hot flushes, decrease vaginal atrophy and positively influence bone health. Hence, they provide an alternative to treat post-menopausal symptoms for patients who have contraindications to hormone therapy [13,14]. As belonging to the polyphenols class, anti-oxidant activity is another important property of phytoestrogens, also related to anti-aging action [15]. The antioxidant efficacy of isoflavones was demonstrated at concentrations equal or lower than the plasma levels of people consuming soy products, thus making this activity physiologically relevant [16]. Protection against cardiovascular diseases has been reported as well, since isoflavones consumption could cause the reduction of LDL cholesterol in hypercholesterolemic patients. This effect seemed to be complemented by the isoflavone ability to restore the endothelial function with beneficial effects on vascular smooth muscle [17]. Phytoestrogens may also positively influence other pathophysiologic

vascular processes, possibly delaying the progression of atherosclerosis. However, discrepancies between the experimental studies demonstrating the vascular benefits of phytoestrogens and the results of clinical trials were observed [18].

Despite the abundance of evidences about health benefits related to phytoestrogens intake, some inconclusive results can be found. Non-standardized animal tests are difficult to compare, and epidemiological studies must be taken with care, since, commonly, many factors can influence the results. For example, the minor incidence of cancer and cardiovascular diseases in Asian populations with respect to Western populations, is frequently ascribed to the higher phytoestrogens intake (due to large soy consumption), but could be due to other food habits as well as environmental factors [3].

As already mentioned, since the 1940s, the estrogenic activity of phytoestrogens has raised preoccupations about possible adverse effects. In particular, the impact on the reproductive development is concerning when the intake of endocrine disruptors occurs in early childhood, since adverse effects may occur even years later, e.g. during puberty [7] and are difficult to be identified without proper follow up of the investigations. As far as anti-carcinogenicity is concerned, contrasting results have been documented; some studies showed potential benefits in prevention of colon, endometrial and ovarian cancer [19], while the role of phytoestrogens in breast cancer development is still not completely elucidated. Suspects are present on negative effects on the growth of pre-existing breast tumours; on the other hand, phytoestrogen ingestion by rodents seems associated with the development of less aggressive breast tumours with reduced metastatic potential [20]. Some data, based on both epidemiological studies and experimental studies on animals, indicate no positive effects or even suspected induction of the tumour [21]. Other possible activities could be reduced fertility and alterations in epigenetic characters and in sexual maturations [22]. As for female reproductive system, exposure to genistein during the development stage showed adverse effects in rodents: these include alterations in ovarian development and function, in the timing of vaginal opening and estrous cyclicity, as well as an increased incidence of uterine adenocarcinoma. As for the effects in males, some epidemiological studies are found about influence of phytoestrogens on men sperm count and characteristics, but results are inconclusive. As for animal studies, once again, contrasting data can be found about the consequences of exposure to isoflavones: some works reported no reproductive defects, others observed abnormalities, such as reduced testicular weight or size, lower testosterone levels and decreased spermatogenesis; however, negative effects seem related to life-long exposure to phytoestrogens [7,23].

Unfortunately, no clear conclusions can be deducted from the large amount of information present on this class of compounds. As already mentioned, this can be ascribed to the complex mechanisms involved in phytoestrogens action, influenced by numerous factors.

1.3 Soy and soy-based food

As introduced in the first paragraph, soy is one of the richest source of phytoestrogens. Soybean (*Glycine max*) is a plant species belonging to the family of *Leguminosae*. The genus name *glycine* was introduced in "Genera plantarum" (1737) by Linnaeus, which observed that among the different species, one had a sweet root (from the greek word "glykós", sweet). However, this name was given to plants which do not actually belong to soy species, although strictly correlated. In fact, soybean was described by Linnaeus only in 1753, with the names *Phaseolus max* or *Dolichos soja*, depending on the specimens; the name "soy" derives from the corruption of the Chinese and Japanese names for soy sauce (Chinese: *sihyàuh*, Japanese: *shōyu*). Soybean is a plant native of China, where it was domesticated probably in the 11th century B.C. [24]; it was later transferred in Korea, Japan and Taiwan [25]. Soy exists in hundreds of varieties, including green, red, white and black beans and it is the most consumed legume in China and Japan, where is used for the preparation of various foods. Soybeans are made up of an 8% of seed coat, a 90% of cotyledon and a 2% of germ. As all legumes, it is a good source of proteins and fiber, but, unlike the others, it also possesses a relatively high amount of fats; in particular, the chemical composition consists in 36% of proteins, 20% of fats, 21% of carbohydrates, 9% of fiber, 9% of water and 5% of ash [26]. The USDA established some quality standards on soy and soy products, considering a range of 30-40% of proteins and a 16-23% of fat as good in commodity-type soybean [27]. As for minor constituents, soy (considered as mature raw bean) is a good source of minerals and vitamins; it is particularly reach in calcium (280 mg/100 g), phosphorus (700 mg/g) and potassium (1800 mg/100 g), while the most concentrated vitamins are niacin, riboflavin, thiamine, pantothenic acid, choline, betaine and α-tocopherol. The complete nutritional profile is shown in Table 2 [28].

Table 2: main constituents of the nutritional profile of soybean (raw, mature).

	Energy	**Proteins**	**Fats**	**Carbohydrates**	**Fibers**	**Water**	**Ash**
Values for 100 g	446 kcal	36 g	20 g	21 g	9 g	9 g	5 g

	Ca	**P**	**K**	**Fe**	**Mg**	**Zn**	**Cu**	**Mn**	**Se**
Values for 100 g	280 mg	700 mg	1800 mg	16 mg	280 mg	5 mg	1.7 mg	2.5 mg	0.02 mg

	Ascorbic acid	Thiamin	Riboflavin	Niacin	Pantothenic acid	Vitamin B6	Folate	Choline	Betaine	α-tocopherol
Values for 100 g	6.0 mg	0.9 mg	0.9 mg	1.6 mg	0.8 mg	0.4 mg	0.4 mg	116 mg	2.1 mg	0.9 mg

Soybean can be used for the preparation of several foods; it is largely employed for the production of oil, which is considered the most used edible oil worldwide. For this purpose, the basic industrial process is based on different steps. If necessary, the beans are dried to reduce moisture content and broken into small pieces; the hull (outer skin) is removed, soy is heated and rolled into thin flakes; hexane (then removed by distillation) is used for the extraction of oil from the flakes. The crude soybean oil is normally processed for edible uses by applying degumming, deodorizing and bleaching procedures. The material which remain after solvent extraction is referred to as defatted flakes and is ground to obtain the so-called defatted soybean meal, which contain approximately 44% of proteins. As for the soybean "skin", it is often used to prepare animal feed. The defatted soybean flakes can also be furtherly processed into soy protein concentrate [29].

Among the other soy-derived food the most common and consumed worldwide are soymilk and tofu. Soymilk is often proposed as an alternative to animal milks; it is also called soy-based drink and originated from Asia, where is a common daily product, especially in China and Japan. It is produced by soaking soybeans in water for about 12 hours at temperatures ranging from 20 to 45°C; then, water is removed and the soaked soybeans are ground to obtain a slurry, which is cooked at 100°C, cooled and filtered to remove the water-insoluble residues [30]. Soymilk is mainly constituted of water (93%), with 3% of proteins, 2% of fat and 2% of carbohydrates [31]. Often vitamins and minerals are added to this beverage, as well as sugars, sweeteners and flavours, to provide better taste and nutritional profile. Tofu originated from China and was later introduced in Japan and East Asia. The production consists of coagulation of soymilk and pressing of the resulting curd. Different coagulants can be used, such as inorganic salts, δ-glucono-lactone and organic acids, which cause protein precipitation and aggregation. There are three main varieties of tofu, based on the moisture content: firm, soft and silken tofu. Soymilk, tofu and other soymilk derivatives are usually consumed as alternative to dairy products by people having intolerance problems or following vegetarian and vegan diets. Another soy-based product which is largely diffused worldwide is soy sauce. It derives from the fermentation of a mixture of soybeans, roasted grain, water and salt. Grain and soybeans are mixed with sodium chloride and molds or yeasts are added for fermentation; after 6-8 months, the resulting product is pressed, and the sauce is pasteurized. It is largely used in Asian countries as a salt substitute and its consumption is recently growing in west countries.

There is a wide range of traditional Asian soyfoods, less known and consumed in the occidental world. Miso is a traditional Japanese paste, used as seasoning and base for soups; first, the so called "*koji*" is obtained by soaking soybeans in water and incubation with conidia of *Aspergillus oryzae*; *koji* is then mixed with steam cooked soybeans and fermented at 30 °C (the traditional fermentation is of 12-24 months, but reduced times are used in industries). Sufu is a Chinese fermented highly flavoured tofu; it is produced by incubation of tofu with *Actinomucor elegans* for 48 h and then ripening for 2 months in presence of a particular dressing mix. Three other products deriving by soybean soaking and fermentation with different microorganisms are natto, tempeh and douchi [32]. Alongside traditional soy-foods, more innovative products are spreading in the international market; they are commonly based on soy flour, soy meal and textured soy protein, mixed with vegetables and cereals. Textured soy proteins are largely used by food industry to resemble meat, since, after rehydration, they have texture and taste similar to beef. These foods are found in the form of burgers, sausages, "meatballs" and cutlets, and proposed as meat-substitutes, for the high protein content.
The consumption of soy-based food has a big variability all over the world. Soybean oil and meal are by far the most used products in the USA, while more traditional soy foods are largely consumed in Asian countries, especially China, Japan and Korea. As far as Europe is concerned, only recently the consumption of soy-based products has grown; the most common are soymilk and soy-based meat substitutes, while among the traditional Asian products only tofu has a moderate diffusion.

1.4 Phytoestrogens analysis in soy food: state of the art

In the latest 20 years, soy foods have raised the attention of the scientific community, because of the high content of phytoestrogens, in particular isoflavones. For many years the studies were conducted by looking at soy-foods as functional, healthy foods, but, recently, the perspective has changed. Investigating phytoestrogens content of soy-food has become interesting for the estimation of the intake by people consuming significative amounts of soy products, with the aim of evaluate a possible concern for health. Several papers can be found in the literature regarding the determination of specific classes of phytoestrogens in food, in particular soy-derived foodstuff. Table 3 summarizes the methods described in the literature for the analysis of phytoestrogens as well as the investigated matrices.

Table 3: summary of the literature methods for the determination of phytoestrogens in food, with particular focus on soy-based products.

SAMPLE TYPE	ANALYTES	EXTRACTION METHOD	INSTRUMENTAL METHOD	REFERENCE
• soy protein • soy foods • nutritional supplements	▪ genistein, daidzein, glycitein ▪ genistin, daidzin, glycitin ▪ malonyl genistin, malonyl daidzin, malonyl glycitin	Solid-liquid extraction (rotary mixer)	HPLC-DAD HPLC-MS Run: 60 min	[33]
• soy flour • tofu • tempeh • textured vegetable protein • soy germ	▪ genistein, daidzein, glycitein ▪ genistin, daidzin, glycitin ▪ malonyl genistin, malonyl daidzin, malonyl glycitin ▪ acetyl genistin, acetyl daidzin, acetyl glycitin	Solid-liquid extraction (stirrer)	HPLC-UV	[34]
• Soybeans	▪ genistin, daidzin, glycitin ▪ malonyl genistin	Ultrasound assisted extraction (UAE)	HPLC-UV C18 column Run: 35 min	[35]
• Soybeans	▪ genistein, daidzein, glycitein ▪ genistin, daidzin, glycitin ▪ malonyl genistin, malonyl daidzin, malonyl glycitin	Pressurized liquid extraction (PLE)	HPLC-DAD e HPLC-MS C18 column Run: 35 min	[36]
• Soybeans	▪ genistein, daidzein, glycitein ▪ genistin, daidzin, glycitin ▪ malonyl genistin, malonyl daidzin, malonyl glycitin ▪ acetyl genistin, acetyl daidzin, acetyl glycitin	Solid-liquid extraction followed by solid phase extraction (SPE)	HPLC-UV C18 column Run: 25 min	[37]
• Soybeans	▪ genistein, daidzein, glycitein ▪ genistin, daidzin, glycitin ▪ malonyl genistin, malonyl daidzin, malonyl glycitin ▪ acetyl genistin, acetyl daidzin, acetyl glycitin	Microwave assisted extraction (MAE)	HPLC-UV C18 column Run: 25 min	[38]
• Defatted soybean meal • soy protein isolate	▪ genistein, daidzein, glycitein ▪ genistin, daidzin, glycitin	UAE	HPLC-DAD C18 column Run: 60 min	[39]
• soybean • beans (various) • lupins • chickpeas • peas • lentils	▪ daidzein, genistein, formononetin, biochanin-A, coumestrol, glycitein, trihydroxyisoflavone ▪ genistin, daidzin	Solid-liquid extraction (homogenizer) followed by SPE	LC-MS/MS C18 column Run: 35 min	[40]
• soybean	▪ genistein, daidzein, glycitein ▪ genistin, daidzin, glycitin ▪ malonyl genistin, malonyl daidzin, malonyl glycitin ▪ acetyl genistin, acetyl daidzin, acetyl glycitin	PLE	HPLC-UV C18 column Run: 45 min	[41]
• fruits and vegetables • meat, fish and dairy products • cereal based food	▪ genistein, daidzein, glycitein, biochanin A, formononetin, shonanin, secoisolariciresinol, matairesinol, coumestrol	UAE followed by hydrolysis and SPE	HPLC-MS/MS Diphenil Column Run: 15 min	[42–44]

• soy dietary supplements	▪ genistein, daidzein, glycitein ▪ genistin, daidzin	UAE	HPLC-UV	[45]
• Cow milk • soy milk • cereal based infant formulae • baby-food	▪ genistein, daidzein, glycitein, biochanin A, formononetin, equol, enterodiol, entero-lactone, secoisolariciresinol, matairesinol, coumestrol, resveratrol, apigenin	Solid-liquid extraction followed by SPE	HPLC-MS/MS C18 column Run: 30 min	[46]
• soybeans • tofu • texured vegetable protein • soy drink • vegetarian sausage	▪ genistein, daidzein, glycitein, formononetin ▪ genistin, daidzin, glycitin	UAE followed by hydrolysis	HPLC-DAD e HPLC-MS/MS C18 column Run: 35 min	[47]
• chickpeas • lentils • beans	▪ genistein, daidzein, glycitein, formononetin, biochanin A ▪ genistin, daidzin, glycitin	QuEChERS	UPLC-MS/MS C18 column Run: 12 min	[48]
• chickpeas • lentils	▪ genistein, daidzein, formononetin ▪ genistin, daidzin	PLE	HPLC-DAD HPLC-MS	[49]
• soy biscuits	▪ genistein, daidzein, glycitein, biochanin A, formononetin ▪ genistin, daidzin, glycitin	QuEChERS	CE-MS Capillary electrophoresis	[50]
• chickpeas • lentils • beans	▪ genistein, daidzein, glycitein, biochanin A, ▪ genistin, daidzin, glycitin	QuEChERS	HPLC-MS C18 column Run: 12 min	[51]
• soybeans • flour • pasta • breakfast cereals • cutlets • tripe • soy drink, • soy nuts • soy cubes • dietary supplements	▪ genistein, daidzein, biochanin A (IS) ▪ genistin, daidzin	Natural deep eutectic solvent extraction (NADES)	UHPLC-DAD C18 column Run: 8 min	[52]
• soybeans, • flour • pasta • breakfast cereals • cutlets • tripe • soy granulate • roasted soybeans, • soy nuts • soy cubes • bread crisps • soy drink • soy supplement	▪ genistein, daidzein, biochanin A (IS) ▪ genistin, daidzin	Ionic liquid based ultrasound assisted extraction (ILUAE)	HPLC-DAD C18 column Run: 13 min	[53]
• soymilk	▪ genistein, daidzein, glycitein ▪ genistin, daidzin, glycitin ▪ malonyl genistin, malonyl daidzin, malonyl glycitin, ▪ acetyl genistin, acetyl daidzin, acetyl glycitin,	Salting out assisted liquid-liquid extraction (SALLE)	UHPLC-ESI-MS C18 column Run: 2.5 min	[54]

• soymilk • environmental samples	▪ genistein, daidzein, glycitein, biochanin A, formononetin, coumestrol, equol, prunetin	Liquid-liquid extraction followed by derivatization	GC-MS	[55]
• cereal based food	▪ genistein, daidzein	Solid-liquid extraction followed by hydrolysis and derivatization	GC-MS	[56]

Raw soybean is the most studied sample, followed by soymilk, defatted soy meal and tofu. Papers regarding the phytoestrogens content of other legumes are common as well, while vegetables, fruit, cereal based food and food of animal origin have been investigated less frequently, since phytoestrogens concentration in these matrices are generally rather low. In the early 2000s the most common extraction technique was the simple solid-liquid extraction or liquid-liquid extraction. A range of solvents is used, the most common being methanol, ethanol, acetonitrile and water, used alone or in mixes; depending on the sample, acidification of the solvents can be useful, in order to favour the analytes solubilization during solid-liquid or liquid-liquid extraction. Agitation during extraction is achieved by shaking, refluxing, stirring, with the aid of vortex, rotary mixer and inversion mixer. Extraction times are usually rather long with this kind of sample preparation (1-2 h), while temperature are commonly set at 25°C, with few works using higher values (up to 90°C) [51]. More rapid and efficient methods were later introduced, with a large employment of ultrasound assisted extraction (UAE). This method guarantees deep contact among the sample and the solvent and exploits the temperature increase due to vibration, reducing time and number of extractions necessary to obtain high recoveries. UAE was employed for a range of soy foods, by optimizing the several variables involved, such as solvent type, temperature, time, ultrasound power and number of extractions. Usually, the same solvents exploited for classical liquid extraction are used, while times are reduced to 15-20 minutes and no more than 2 consecutive extractions are normally carried out. Other advantageous techniques introduced in the late 2000s are microwave assisted extraction (MAE) and pressurized liquid extraction (PLE), which, analogously to UAE, exploit different physical effects to enhance extraction efficiency. Solid phase extraction (SPE) have been largely utilized for sample purification and enrichment. This method is particularly useful if matrix effects are expected and low concentration of the analytes makes it necessary to perform a pre-concentration step. SPE is normally applied following solvent extraction for solid samples. It is especially employed in combination with sensitive instrumental techniques, which generally need a clean up of the samples, to avoid excessive matrix effects and high noise signal. For example, SPE was used for a general method applied for the determination of phytoestrogens in a huge range of food samples, including vegetables, meat, cereal based products, dairy products, with analysis performed by LC-ESI-MS [42–44]. Recently, innovative methods have been described for the extraction of phytoestrogens; among them,

QuEChERS (Quick Easy Cheap Effective Rugged and Safe) methodology proved to be a valid choice for the treatment of legumes and soy-flour based biscuits for subsequent determination of isoflavones. This technique is versatile, and modifications of the original version proposed for fruit and vegetables make it suitable and adaptable to a range of applications. Other pioneering techniques have been recently proposed for the treatment of soy-based products, such as natural deep eutectic solvent (NADES) extraction, ionic liquid-based ultrasound assisted extraction (ILUAE) (for solid samples) and salting-out assisted liquid-liquid extraction (SALLE) (for liquid samples); they proved to be effective and "green", but their application remains limited.

Concerning the instrumental techniques, analysis of phytoestrogens is mainly performed by high performance liquid chromatography (HPLC); when high concentrations are expected the detection is commonly achieved by UV or diode array detectors (DAD). HPLC coupled to mass spectrometry is also used, especially if higher sensitivity and specificity are required. A few examples of gas chromatography (GC) coupled to mass spectrometry are found in the literature for determination of phytoestrogens in food matrices [55,56]; in fact, these analytes require a derivatization step before GC analysis. For this reason, HPLC is the most common choice for phytoestrogens analysis.

Among the wide class of phytoestrogens, the substances which raise the major interest are isoflavones. Isoflavones are present in soy as aglycones and in glycosylated form; daidzein, genistein, glycitein and the corresponding glucosides, e.g. daidzin, genistin and glycitin are by far the most studied analytes, since their concentration in soy food are generally at the ppm levels; sometimes, the acetyl and malonyl derivatives of daidzin, genistin and glycitin are quantified as well. Other compounds, such as biochanin A and formononetin are less investigated, being their concentration in soy at lower values. Few papers reported the determination of isoflavones metabolites in food matrices of animal origin; in fact, equol, enterodiol and enterolactone can be found in meat and dairy products, since they are produced in the intestine by bacterial action, after isoflavones intake.

Analysis of lignans and coumestans is less frequent if compared to isoflavones, and usually they are determined in cereals and other vegetables, rather than in soy. Compounds that are commonly quantified are secoisolariciresinol, matairesinol, resveratrol and coumestrol.

Quantitation of several substances in food matrices is a challenging task; recovery and matrix effect of the extraction procedure as well as the instrumental analysis need to be optimized. In fact, obtaining satisfactory extraction efficiency and optimal sensitivity for a high number of analytes is not straightforward and strictly dependent on the sample complexity. In the reported literature, method development is usually performed by optimizing the various steps and variables one at a time. Only two examples of the application of the multivariate approach (experimental design) have been described for the quantitation of isoflavones in soy-based foods [52,53].

1.5 Aim of the thesis

Because of the numerous studies which show conflicting results, it is necessary to deepen the knowledge about human exposition to phytoestrogens, pinpointing the sections of population exposed to higher risk. In fact, as already extensively discussed, the effects of phytoestrogens are multiple, as well as strictly correlated with the single individual characteristics, namely the exposition to other chemicals, gender, age and genetics. The possibility that long-term exposition to high doses of phytoestrogens could lead to negative consequences in certain individuals requires a careful evaluation of daily intakes. The increase in the occurrence of allergies and intolerances as well as the growing diffusion of vegetarian and vegan diets caused the modification of the eating habits of many people, which introduce in their diet soy food to replace other products. Several soy-based preparations are presented as meat and dairy products substitutes and the consumption of many servings per day could lead to take high phytoestrogens doses, comparable to the ones prescribed to women for menopausal symptoms alleviation. Hence, the phytoestrogens daily intakes derived from the consumption of soy-based food must be carefully evaluated, thus requiring the quantitation of these compounds. In fact, even though the environmental presence of phytoestrogens has been reported, food remains the major route of human exposition.

In this framework, analytical chemistry plays a key-role to obtain reliable information about the phytoestrogens content of soy-based food. Since the most common soy food consumed in Italy are soy-milk and soy-based burgers, the PhD main work is focused on these two matrices. In order to attain reliable data, accurate and precise quantitation is fundamental, thus making the development and optimization of the analytical strategies essential.

The first work was based on the development of a GC-MS method for phytoestrogens determination in soy-milk. Then, an LC-MS method was studied, and the two were compared for phytoestrogens determination in soy-based drinks. The LC-MS strategy was later improved and applied to another soy-based matrix, namely soy burgers. Different extraction procedures on this matrix were tested and compared. The more innovative one, i.e. the QuEChERS methodology was further investigated to achieve optimal performances. Finally, the optimized technique was applied to phytoestrogens determination in a wide range of soy-burgers form the Italian market. The multivariate approach of experimental design was applied during the various stage of the methods development, especially for optimization of sample pre-treatment.

The described research line was followed on the basis of accurate literature study. In fact, the selected analytes included two fundamental isoflavones present in soy (genistein and daidzein) and three other substances which were rarely quantified in soy-based product (formononetin, biochanin A and

coumestrol), in order to improve the knowledge on the phytoestrogens content of soy-based products. Only a few GC-MS methods are found in the literature and they are characterized by long sample preparation (due to the derivatization step). Therefore, it was necessary to improve the GC-MS analysis as well as provide a direct comparison with an LC-MS method. This information could be interesting and useful for laboratories where the more expensive LC-MS instrumentation is not available. As for experimental design, the employment of this chemometric tool for procedure optimization remains limited; hence, the use of this approach during the various stages of the work represents a significant innovation. Finally, the quantitation of the five analytes in several samples belonging to a novel food matrix (soy burgers), provides valuable and new information, putting the basis for the risk assessment of specific classes of consumers.

Chapter 2

Instruments and techniques

2.1 Chromatographic techniques coupled to mass spectrometry

Hyphenated techniques are doubtless among the most powerful and versatile analytical methods. The term hyphenated derives from the word “hyphen” (-) indicating that two techniques are joint together and expressed as the respective names separated by a dash. Normally, two complementary techniques are combined, in order to obtain the best information and performances. The most common and successful coupling used in analytical chemistry is between chromatography and mass spectrometry (MS). This union provides a wide range of advantages both for quantitative and qualitative analysis, especially when complex mixtures must be analysed, joining the separation capacity and versatility of chromatography, with the potential of mass spectrometry detection. Both gas chromatography (GC) and liquid chromatography (LC) can be coupled to mass spectrometry (MS), thus covering an extensive range of applications. The main properties of these techniques are [57]:

- No alteration of the chromatographic resolution by the mass spectrometry detector
- High sensitivity
- Possibility to identify and/or confirm eluted compounds

- Universality, i.e. ability to detect all classes of chemicals (if we consider the union of GC-MS and LC-MS)
- Production of a signal proportional to concentration
- Possibility to deconvolute unresolved chromatographic peaks
- Minimum interferents presence if selective detection modes are used

Moreover, some mass analyzers (or combined analyzers) provide the possibility to perform tandem mass spectrometry, which even enhances sensitivity and specificity of the detector. Thanks to its characteristics, MS is considered the instrumental technique which can provide the largest amount of chemical information using the smallest quantity of sample; therefore, it is doubtless one of the most powerful tools for the modern analytical chemist [58].

The described features make chromatographic-mass spectrometric methods the most suitable for the aim of this thesis, and therefore were selected for analytes determination in the different parts of the work, after proper studies and optimizations.

2.1.1 GC-MS

Gas chromatography was historically the first technique coupled to mass spectrometry; this because, when capillary columns are used, the low gas flow (around 1 mL min^{-1}) provided by a gas chromatographic analysis permits an easy coupling with the under-vacuum mass spectrometer, with the aid of a pumping system. Moreover, the species coming from the column are in the gas phase, which is the required condition for the ionization process in the mass spectrometer source.

A GC-MS analysis involves the injection of a small volume of sample (usually 1 µL), which can be subjected or not to splitting, namely the reduction of the injected volume (splitless mode is preferred when trace analyses are performed); the injector can be set at constant temperature, or a programmed temperature vaporization can be chosen if a particular configuration is available (PTV injector). The application of a temperature gradient during the injection phase can enhance the quantitative transfer of species with high molecular mass to the column, as well as improve the repeatability. Then, the chromatographic run allows the separation of the compounds through a thermal gradient; finally, the eluted chemicals arrive to the ion source by passing in a heated transfer line, for subsequent mass spectrometric detection.

The most common ion source used in GC-MS is the electron impact source (EI) which provides high energy ionization and therefore permits molecules fragmentation; hence, structural elucidations or unknown identifications, through the use of spectral libraries, are possible. In fact, with a fixed electron energy, reproducible mass spectra are normally obtained, and databases can be used for

compounds identification. For the mechanisms involved, this method provides only positive ionization.

Among the different GC-MS configurations, the instrument used in this doctorate project was a GC coupled to an ion trap analyzer. A 3-D ion trap is made of a central ring electrode and two conical endcaps; an electric quadrupolar field is generated and permits the trapping of ions of certain m/z values depending on the radiofrequencies applied. This analyzer allows to perform tandem MS experiments in time; in fact, precursor ions of specific m/z can be selectively trapped, fragmented through collisions with an inert gas and, subsequently, only selected product ions can be detected. By applying tandem MS, specificity is greatly improved, and background noise is reduced to a minimum.

One of the drawbacks of the use of GC-MS is that not all molecules are volatile and analysable by this technique. An approach usually followed to overcome this problem is to perform derivatization, which allows to substitute functional groups on the molecules, forming derivative species that can be analysed by gas chromatography. Moreover, usually, the compounds must be stable at the high temperature reached during the chromatographic run (up to 310 °C). On the other hand, GC-MS is usually cheaper, requires less maintenance with respect to LC-MS systems and has the great advantages of high chromatographic resolution and negligible ion suppression, thus limiting problems of matrix effect.

2.1.2 HPLC-MS

The coupling of liquid chromatography, in particular HPLC (high performance liquid chromatography) systems, with mass spectrometry was initially tricky. In fact, when liquid elution is performed, a huge volume, and therefore pressure, comes out of the chromatographic column. To face this problem, during the years a range of different interfaces were introduced, such as particle beam, fast atom bombardment (FAB), atmospheric pressure photoionization (APPI), atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI).

The ESI source is by far the most used among the atmospheric pressure interfaces because it provides the widest applicability. The HPLC-MS analysis consists of the injection of 10-20 µL of sample, separation onto a packed column, by an elution gradient (commonly reversed phase chromatography is used) and nebulization of the eluate in the ESI chamber. Here, with the aid of a gas flow, the solvent is evaporated, molecules are ionized by complex mechanisms and transported into the mass spectrometer by applying a proper voltage between the spray needle and the capillary at the entrance of the analyzer. Both positive and negative ionization are possible and achieved by losing or gaining a proton, but also by the formation of adducts. ESI is a soft ionization technique, therefore, normally, no in-source fragmentation is observed.

Different analyzers are used in LC-MS coupling, giving different performances in terms of mass resolution and sensitivity. The instrument used during the thesis was an HPLC coupled to a triple quadrupole. This is the preferred configuration when reliable quantitative analyses and high sensitivity are required. In fact, the triple quadrupole allows to perform a range of scan modes to optimize the sensitive detection of target compounds. Tandem MS experiments are performed in space, with the selection of the precursor ion in the first quadrupole, the fragmentation in the collision cell, and the detection of selected product ions in the second quadrupole. In particular, the multiple reaction monitoring (MRM) mode is the most appropriate in quantitative analysis. The selection of a precursor ion and normally two product ions for each molecule (quantifier and qualifier ions), as well as the optimization of fragmentation parameters, permits to maximize specificity and obtain low detection limits. The number of applications of LC-MS is definitely larger than GC-MS; nevertheless, the complex phenomena occurring in the ESI source give raise to problems related to matrix effect. Therefore, the methods for the analysis of complex matrices should be carefully optimized, also in terms of pre-treatment of the samples.

2.2 Extraction and purification techniques

In an analytical method, the pre-treatment step is as important as the instrumental analysis and should be carefully evaluated on the basis of the considered samples. Food matrices are usually quite complex, because of the numerous constituents, and obtaining clean extracts and quantitative recoveries is not straightforward. A wide range of techniques are proposed nowadays to treat solid and liquid food samples. Among them, three methods were used in the different parts of the work, selected for their feasibility and ease of use: ultrasound assisted extraction (UAE), solid phase extraction (SPE) and the Quick, Easy, Cheap, Effective, Rugged and Safe methodology (QuEChERS).

2.2.1 Ultrasound assisted extraction

Ultrasound assisted extraction (UAE) is one of the most diffused techniques for extractions of solid samples. It is easy and versatile, since the most appropriate solvent can be chosen, based both on the analytes of interest and on the type of matrix. Normally, the solid sample is ground and homogenized and extraction is achieved by placing the sample in an ultrasonic bath, after the addition of a proper solvent or mixture of solvents. The variables usually involved in this type of extraction are solvent, extraction time, temperature, number of extractions and ultrasound power.

The advantage of using this technique is given by the action of the ultrasounds on the sample particles: the ultrasound mechanical effects causes the disruption of biological cell walls, allowing a penetration of the solvent into cellular materials and the release of the cell content; in addition, ultrasounds improve the mass transfer from the sample to the solvent thanks to the micro-streaming effect (vibration of small gas-filled bubbles inside the material) [59]. The action of ultrasounds permits to significantly accelerate the extraction of organic compounds from different matrices, thanks to the more efficient contact between the solid and the solvent. Both the increase of pressure and temperature caused by the vibrations favour solvent penetration and improve solubility and diffusivity. For medium polarity substances, several solvents can be used, such as water, methanol (MeOH), acetonitrile (ACN), ethanol, also in mixtures and at different pH, based on the properties of the analytes which must be extracted. The time of extraction depends on the concentration levels, and again on the sample characteristics. The variables involved in the ultrasound extraction are strictly intercorrelated and must be properly optimized for the purpose. Commonly, to obtain high recoveries, more than one extraction is performed, by centrifuging the sample, collecting the extract and adding more solvent to the solid residue for the following extraction. Once again, the choice of the number of extractions depends on the case, and usually a compromise between rapid and efficient extraction must be found. UAE can be combined with other techniques, and followed by a clean-up step, especially when the presence of interferents and matrix effects are likely to occur.

2.2.2 Solid Phase Extraction

Solid phase extraction (SPE) is a technique which has found a huge development since its introduction in the 1980's. It currently remains one of the most used strategy for the preparation of a wide range of biological, environmental and food samples. SPE is mainly used for extraction, matrix purification and analytes pre-concentration [60]. It can be used directly on liquid samples, to simultaneously perform analytes extraction and clean-up, or applied as a purification step after solid liquid or liquid-liquid extraction. The principle of solid phase extraction is based on chromatography; in fact, the SPE procedure involves the use of a stationary phase to bind the analytes of interest, and an elution with a specific mobile phase, after eliminating matrix interferents. The most common configuration for SPE are plastic or glass cartridges, filled with a certain amount of sorbent and used in combination with vacuum systems, to force the passage of liquids through the densely packed material. Commonly, the procedure involves the following main steps:

- Conditioning of the solid phase (activation of the binding sites)
- Load of the liquid sample or extract
- Wash of the cartridge from interferent unbound species

- Elution of the analytes with a proper solvent

The classical approach involves the operator in each stage of the procedure, but, recently, also automated systems have been developed, usually directly coupled with chromatographic instruments. Over time, a large selection of formats, sorbents and dimensions have been proposed, to respond to the different sample preparation needs. The technique is versatile and permits the washing of interferents and selective elution, by using the most appropriate solvent or mixture. Some drawbacks of this technique are the long time required when large volumes of sample must be loaded, and the difficulties encountered to obtain reproducibility in the loading/eluting speed, which can largely influence the absorption/desorption process. As always, the method should be optimized for each kind of sample and analytes; the solvents and volumes used in the different stages of the procedure must be carefully chosen and tested, to obtain optimal recoveries and clean-up.

2.2.3 QuEChERS

QuEChERS is the acronym for Quick, Easy, Cheap, Effective, Rugged and Safe, and it is related to an extraction and purification technique introduced in 2003 by Anastassiades at al [61]. It involves a first extraction step with a mixture of water and acetonitrile (ACN), phase separation through the addition of salts and a final clean-up of the organic phase, through dispersive solid phase extraction. The original procedure was conceived for pesticide analysis in fruits and vegetables at the trace levels. The following steps were involved:

- fruit or vegetables homogenized sample put in a plastic centrifuge tube
- Addition of ACN and vigorous shaking for 1 min by using vortex
- Addition of anhydrous $MgSO_4$ and NaCl, and immediate shaking for 1 min
- Centrifugation and collection of the organic layer
- Dispersive-SPE clean-up on the ACN extract with PSA (primary secondary ammine) sorbent and anhydrous $MgSO_4$
- Hand or vortex shaking for 30 s
- Centrifugation, filtration of the supernatant and GC-MS analysis.

The general procedure is shown in Fig. 5.

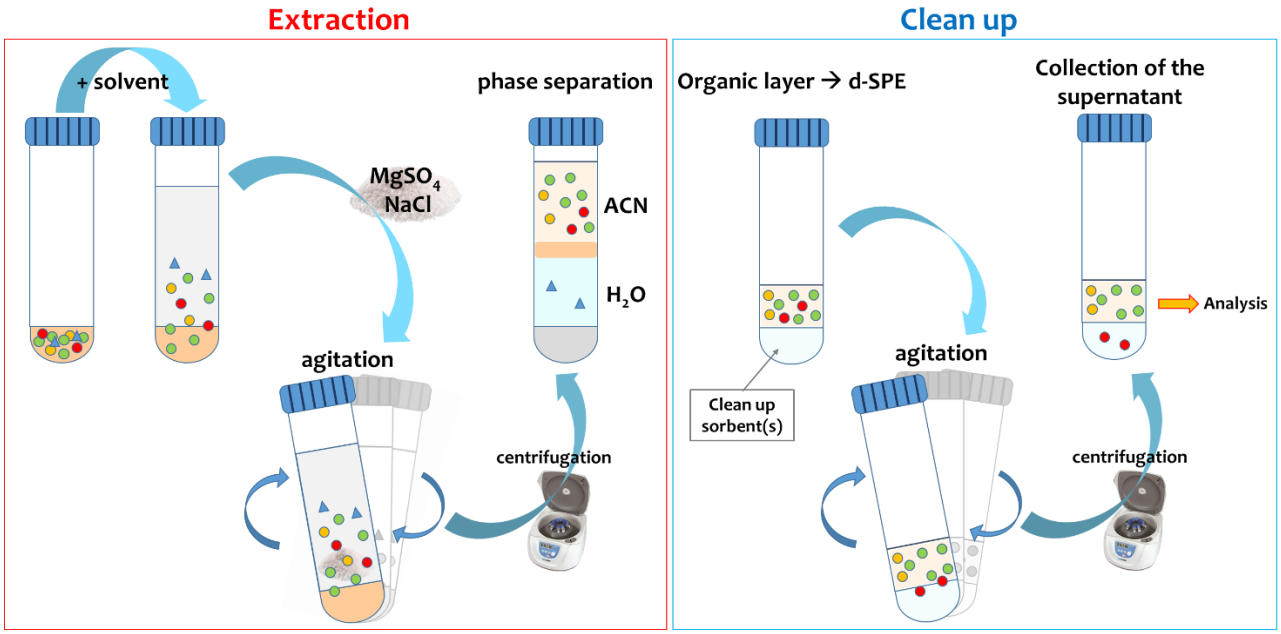


Figure 5: scheme of the different steps involved in a QuEChERS procedure.

The method permits the rapid and efficient extraction of a wide range of medium polar analytes, by excluding interferents in two different steps: the most polar ones are eliminated by their solubilization in the aqueous phase after salts addition, while the others are trapped in the clean-up sorbent. Moreover, the salting out effect which occurs by adding a high amount of salts to the solvents mix, favours the dissolution of the analytes of interest in the organic phase, providing effective extraction. This approach is cheap, does not require particular or expensive instrumentation, and allows preparation of a high number of samples in short times.

Thanks to the several advantages, during the years, the QuEChERS approach has been applied for the analysis of a broad spectrum of analytes in numerous food and environmental samples. This was possible thanks to the possibility of customizing the procedure, by choosing the solvent mix, salt formulation, buffer addition and by selecting the proper clean-up sorbent or mix of sorbents [62].

The QuEChERS application implicates a great number of variables, since several steps are involved in this technique and a huge range of combinations are possible. Therefore, the various options must be evaluated based on each particular application and the optimization phase requires particular care.

2.3 Chemometric techniques

Chemometrics is a branch of analytical chemistry, which exploits statistical and mathematical tools both to obtain good quality chemical data and to extract the maximum information from already acquired data. The term chemometrics was coined in 1971, although the basic methods employed were conceived from the beginning of the century. Nevertheless, still nowadays, useful chemometric techniques are not well established in the workflows of chemical laboratories. A fundamental aspect of chemometrics is the application of the multivariate approach, namely the consideration of all the variables involved in chemical systems or data, as to have an overall view and to better use good quality data. In fact, when objects are related to a large set of variables, data analysis and interpretation could be difficult and the aid of chemometric tools becomes essential. Besides its capacity to improve the achievable information from collected data, chemometrics is able to drastically reduce times and costs of experiments and data analysis. Chemometric methods include unsupervised visualization techniques (Principal Component Analysis, clustering), multivariate calibration and regression (Principal Component Regression, Partial Least Squares…), classification methods and experimental design. During this thesis, experimental design and principal component analysis (PCA) were employed, also in combination.

2.3.1 Experimental Design

Experimental design (or design of experiments, DOE) is a multivariate approach, whose scope is to rationally plan and select the experiments to perform, in order to attain the best knowledge of a chemical system or process with the minimum experimental effort. A chemical problem (such as a chemical reaction, an extraction procedure, an instrumental analysis…) is characterized by variables and responses: variables (or factors) are entities which can be set at chosen values, independently one from another; the response is a measurable quantity which indicates the result of an experiment. Variables are changed in specific ranges, which joint together define the so called experimental domain; in other words, the domain is the n-dimensional space which encloses all the values of the n variables investigated [63]. Design of experiment allows to understand the effects of variables on one or more responses and to obtain optimal conditions within the studied experimental domain. It is commonly used as the optimization strategy opposed to the one-variable-at-a-time (OVAT) approach. When a process depends on several variables it is normal and common that these variables are somehow dependent the one from the others, i.e. interactions are present. The best setting of a certain variable depends on the surrounding conditions, namely the other variables involved [64]. In chemistry, it is rather unusual that a system depends on a single variable; commonly, many variables

influence the response. When performing an OVAT "optimization", one variable is changed, when all the others are kept constant, in order to understand the effect of each one separately, in an apparently simple way. Once the best value of a variable is defined, it is set, and the following variables are studied in the same way. Working in such a manner normally leads to find only local optimal conditions; in fact, a small part of the experimental domain is explored, and the interactions among variables are completely ignored. Moreover, when a large number of factors has to be evaluated, a huge number of experiments are required to satisfactorily explore the domain and reach the desirable results.

On the other hand, experimental design is a powerful tool to investigate the effect of the variables on a response in a simultaneous way and to predict the response of experiments in conditions that were not tested. In fact, the most commonly used designs allow to obtain a model after experiments performance: this model expresses the response as a function of the variables (each variable will have a coefficient) and, depending on the design, can contain linear, quadratic and interaction terms. To obtain reliable optimization it is important to choose the right model (right design) which can approximate the system under study, as well as select the proper variables and experimental domain. Since it is not always straightforward to choose the right variables and domain to investigate, a deep knowledge of the procedure to optimize is needed, both thanks to theoretical principles, as well as preliminary tests.

The following steps should be accomplished when performing an experimental design [65]:

- Definition of the problem and the goal of the experiments
- Selection of the response(s) to be measured
- Detection of all the variables possibly influencing the process
- Choice of the experimental domain
- Planning of the experiments (construction of the appropriate experimental design, based on the goal and the model which must be computed)
- Performance of the experiments and data acquisition
- Elaboration and data analysis (model computation).

The choice of the design depends on the goal and on the level of depth needed to understand the problem and consequently optimize the response. Experimental designs can be divided in three main categories:

1. Screening designs
2. Response surface designs
3. Mixture designs

The first two types are usually employed sequentially for optimization purposes, while the last one is used in the particular case of mixture variables (components), namely variables which are not independent (the sum of their values must be 1 or 100%).

Among the aims of this thesis, procedure optimization plays an important role; sample pre-treatment is commonly made up of a series of steps, depending on several variables, and its optimization is fundamental to reach good figures of merit of the entire analytical method. In order to optimize sample preparation procedures, for example derivatization and extraction of the analytes of interest, both screening designs and response surface designs have been applied at different stages of the research.

2.3.1.1 Screening designs

When several factors potentially influence a response, performing experimental design might lead to a large number of experiments, although less than what is reached following the OVAT approach. Nevertheless, rarely more than 3 or 4 factors are truly significant in affecting the response, therefore the optimization should be limited to these ones. Screening designs are ideal to detect the influent variables with a reasonable experimental effort. They can be used either to understand linear effects and interactions (full factorial designs), or to screen a wide number of factors with a limited number of experiments (Plackett-Burman designs). An important feature of screening designs is that they can be used to explore the effect of both quantitative and qualitative variables at the same time [66].

The simplest type of experimental design is the full factorial [65,67], which can be considered a screening design. It is used to build a model which expresses the response as a linear function of the variables, including linear terms and first order interactions; it allows to identify which factors have a significant effect on the response as well as to understand if the variation of a factor can influence the effect of the others (presence of interactions among variables). The general equation of the model obtained by a 2 levels full factorial design is the following:

$$Y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{1 \le i < j}^{k} b_{ij} x_i x_j$$

where Y is the response, k is the number of the variables, x_i are the variables, b_0 is the constant term, b_i are the coefficients of the variables and b_{ij} are the coefficients of the interactions. For instance, in the case of 2 factors, the model would be: $Y = b_0 + b_1 x_1 + b_2 x_2 + b_{12} x_1 x_2$.

Since the model is linear, this design is usually not employed for optimization purposes, because it only permits to find a "direction", where to move to search for the real optimum. In fact, only quadratic models can theoretically pinpoint a real maximum or minimum of a specific response. Nevertheless, it is a useful tool to solve problems which could be difficult to understand by using an

OVAT approach. In the full factorial design, the factors are investigated at two levels, which are coded as -1 e +1 (or simply – and +); usually, a third level, at the centre of the variables' ranges is introduced and corresponds to a coded 0 value. The number of experiments of a full factorial design is 2^k, where 2 indicates the levels of the factors and k the number of factors. The experimental matrix, made by 2^k rows and k columns, is easily built by alternating in an appropriate way the -1 and +1 levels and obtaining all the possible combinations of the factors' levels; as an example, Table 4 reports the matrix for a 3 factors design.

Table 4: experimental matrix for a full factorial design with 3 factors and 2 levels.

Experiment	Factor 1	Factor 2	Factor 3
1	-1	-1	-1
2	+1	-1	-1
3	-1	+1	-1
4	+1	+1	-1
5	-1	-1	+1
6	+1	-1	+1
7	-1	+1	+1
8	+1	+1	+1

Once the matrix is prepared, it is of fundamental importance to perform the experiments in a random order. This is useful in every experimental design to avoid systematic errors or drifts, which could lead to wrong conclusions.

Normally, at least 3 replicates of the central point of the experimental domain (all factors set at the 0 level) is used to estimate the experimental variance associated with the performance of the experiments. Thanks to this estimation, it is possible to determine the statistical significance of the factors involved: if the effect of a factor on the response is smaller than the experimental variability, it probably possesses a non-significant effect, since variations given by casual errors influence the response more than the variation of the factor. More precisely, given the experimental standard deviation, calculated over the n_c replicates of the central point, we can establish the significance of each variable, by comparing its coefficient to a confidence interval associated to the 95% of probability. A factor is statistically significant if the following condition is satisfied:

$$|b_i| > t_{0.05,Dof} \cdot \frac{s}{\sqrt{2^k}}$$

where b_i is the coefficient of the i-variable, $t_{0.05,Dof}$ is the t-student variable associated to the degrees of freedom of the replicates (n_c-1) and to the 95% confidence level, and s is the experimental standard deviation (square root of the variance). Therefore, by performing a simple full factorial design, we

are able to establish the significance of the factors and the presence of interactions among them, thus giving an indication of which design to perform next, in order to find the real optimum. Moreover, the results of a first set of experiments in a defined domain allow to understand if the selected variables' ranges should be varied, in order to move to a more appropriate experimental domain where to look for the optimal conditions.

Despite its usefulness, it is not always possible to perform a full factorial design. This is because the number of experiments exponentially grows with the number of factors; if 32 experiments (5 factors investigated) may still be feasible, depending on the considered chemical problem, with a number of factors greater than 5, things get complicated. One option to reduce the number of experiments in screening, is to apply a fractionated factorial design. This design is a reduced factorial where some main effects are "confounded" with minor interactions, supposing these interactions not significant. Depending on how many factors are confused with interactions, a 2^k design is reduced to a 2^{k-x} design, determining an important saving in experimental effort. In performing a fractionated factorial design, it is important to eliminate some experiments but to maintain a balanced design.

A powerful alternative to fractionated factorial design in screening studies is the Plackett-Burman design, proposed in 1946 [68]. It permits to examine a large number of factors with a small number of trials and to determine the main effects of these factors, i.e. the linear terms of the postulated model [69], as shown by this function:

$$Y = b_0 + \sum_{i=1}^{k} b_i x_i$$

where Y is the response, k is the number of the variables, x_i are the variables, b_0 is the constant term and b_i are the coefficients of the variables.

As the factorial designs, two levels (low and high) are set for each variable, coded as – and +. The peculiarity of this design is that, considering k factors, the number of experiments is equal to 4n, where 4n is the first multiple of 4 greater than k; thus, with 8 experiments we can study up to 7 factors, with 12 experiments up to 11 factors and so on. The construction of the experimental matrix for a Plackett-Burman design is quite simple. It is based on the cyclic permutation of the first row, which can be found in the literature for a defined number of experiments; in fact, in their paper, Plackett and Burman presented the first row of designs of a number of experiments up to 100. The first rows for designs with a number of experiments from 8 to 24 are illustrated in Fig. 6.

$N = 8.$ + + + − + − −

$N = 12.$ + + − + + + − − − + −

$N = 16.$ + + + + − + − + + − − + − − −

$N = 20.$ + + − − + + + + − + − + − − − − + + −

$N = 24.$ + + + + + − + − + + − − + + − − + − + − − − −

Figure 6: First row of the experimental matrix for Plackett-Burman designs of 8, 12, 16, 20 and 24 experiments (N=4n).

The cyclic permutation of the first row consists in the shifting of the row of one place to obtain the following one, 4n-2 times; the matrix is finally completed by adding a row of minus. In such way a balanced design is obtained, in which each factor is equally investigated at the low and high level (same number of experiments at the – level and at the + level for all factors). An experimental matrix for a 12 experiments Plackett-Burman design is shown in Fig. 7, which highlights the shifting of the first two rows as an example.

Exp	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}
1	+	+	-	+	+	+	-	-	-	+	-
2	-	+	+	-	+	+	+	-	-	-	+
3	+	-	+	+	-	+	+	+	-	-	-
4	-	+	-	+	+	-	+	+	+	-	-
5	-	-	+	-	+	+	-	+	+	+	-
6	-	-	-	+	-	+	+	-	+	+	+
7	+	-	-	-	+	-	+	+	-	+	+
8	+	+	-	-	-	+	-	+	+	-	+
9	+	+	+	-	-	-	+	-	+	+	-
10	-	+	+	+	-	-	-	+	-	+	+
11	+	-	+	+	+	-	-	-	+	-	+
12	-	-	-	-	-	-	-	-	-	-	-

Figure 7: experimental matrix for a 12 experiments Plackett-Burman design. The rows are the experiments while the columns are the investigated factors (X_1-X_{11}).

Although the number of factors can be theoretically equal to 4n-1, it is common practice, if possible, not to use the smallest design as possible, but to perform more experiments, introducing the so called dummy factors. If we use a design with the minimum number of experiments, we will obtain a saturated design; this means that all the experiment responses are used to compute the coefficients of the model and no degrees of freedom (DoF) are available. In fact, if we must determine the effect of 7 factors, 8 runs will be necessary to estimate the 7 coefficients of the factors and an 8^{th} coefficient which is the constant term. The lack of degrees of freedom makes it difficult to estimate the significance of the factors studied, although some practical considerations can be used to identify important factors (for example, looking at the average effect of the variables, and considering significant only the ones which possess an effect larger than the average). On the other hand, if the

number of factors k is smaller than 4n-1, it is possible to compute the real terms of the model plus a certain number of coefficients associated to the effects of the dummy factors. These factors are unreal and consequently associated to non-significant effects on the response. The significance of the other factors can be determined by means of a simple comparison among coefficients, choosing as significant the factors with a coefficient higher than the ones of the dummy factors. In alternative, a better evaluation can be made by comparing the coefficients with the usual confidence interval associated to the 95% of probability. Dummy factors' coefficients are used to calculate the experimental variance, with the following formula:

$$s^2 = 4n \cdot \left(\sum_{i=k+1}^{4n-1} {b_i}^2 \right) / (4n - k - 1)$$

where s^2 is the experimental variance, $4n$ is the total number of experiments, k is the number of factors and b_i are the coefficients of the dummy factors ($k+1 \leq i \leq 4n-1$). Hence, a factor is considered significant if the usual condition is satisfied:

$$|b_i| > t_{0.05,Dof} \cdot \frac{s}{\sqrt{4n}}$$

where b_i is the coefficient of a factor, $t_{0.05,Dof}$ is the t-student variable associated to the degrees of freedom of the design (equal to the number of dummy factors) and to the 95% confidence, and s is the experimental standard deviation (square root of the variance). For instance, if we have 7 factors and we choose a design with 12 experiments, 4 dummy factors will be included, and we will be able to estimate variability with 4 degrees of freedom, providing a more reliable evaluation of the significance of the variables.

Even though this kind of design could be the best choice to study complicated processes, where a lot of variables are involved, some drawbacks are present. First of all, in order to perform an effective screening, the experimental domain should be chosen carefully; this choice is not always straightforward, because the difference from the low and the high level of a factor has to be large enough to detect a possible effect (not confused with experimental variability) but not huge, in order to avoid any masking of the other effects [70]. Moreover, the Plackett-Burman designs are not able to detect interactions among the variables and misleading conclusions are possible if a main effect is masked by an interaction. Nevertheless, screening design are sometimes mandatory, and are a simple and rapid approach to rationally select the most important factors to be further investigated.

2.3.1.1 Response Surface design

When we want to optimize a procedure, usually linear models are not sufficient to describe the relationship between the variables and the response. In fact, they only permit to identify a direction of maximum or minimum response and, in the case of continuous quantitative variables this information does not necessarily lead to a real identification of the so called "sweet spot" (optimum). Designs which allow to build quadratic models are recommended for this purpose. They are based on the investigation of at least 3 levels of each variable, in order to detect curvature in the response surface. Since the response surfaces are continuous functions, only quantitative variables can be studied by this kind of design and the function obtained by modelling the response is the following:

$$Y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{1 \le i < j}^{k} b_{ij} x_i x_j + \sum_{i=1}^{k} b_{ii} {x_i}^2$$

where Y is the response, k is the number of the variables, x_i are the variables, b_0 is the constant term, b_i, b_{ij}, and b_{ii} are the coefficients of the first order terms, of the interactions and of the second order terms, respectively. Usually, no more than 3 or 4 variables are considered in this kind of design, since the number of experiments exponentially grows with the number of factors; this is because more coefficients must be computed to obtain quadratic models and a certain number of degrees of freedom are necessary to have a reliable statistical evaluation of the models. Moreover, from 3 factors on, we cannot visualize the overall surface; hence, graphical evaluation and identification of maximum or minimum responses are difficult.

The workflow for a multivariate optimization strategy by response surface methodology (RSM) is the following:

- Selection of the most important factors (either by a screening design or previous knowledge)
- Selection of the appropriate response surface design
- Construction of the model from the design results
- Statistical evaluation and validation of the model
- Identification of the conditions for the optimal response
- Test of the optimal experiment

If the responses to optimize are more than one, commonly a compromise must be chosen, and particular strategies (such as desirability function [71] or pareto fronts) are followed to select the best solution and hence have all responses at an acceptable value.

When using RSM it is important to choose a design that is suitable for the aim of the experimenter. Response surface designs are characterized by features that can be useful to make the right choice.

First of all, the number of experiments to perform is slightly different depending on the design, and, in the case of expensive procedures, even a reduction of one trial can make the difference. The same consideration applies for the selection of the replicates in the centre point; triplicate experiments are considered a minimum, but a higher number should be chosen if possible, in order to have a better estimation of experimental variability. In addition, two important characteristics should be taken into account when selecting a design: orthogonality and rotatability [72]. A design is orthogonal if the covariance of the coefficients computed in the model is zero, that is to say that the computation of a coefficient is independent from the computation of the others. Orthogonality is of fundamental importance if we want to use reduced models. Reduced models are models in which the terms that were not significant are removed; this action is correct when orthogonality is satisfied. As for rotatability, a design is rotatable if the leverage (or variance function) is symmetrical with respect to the centre of the design (domain); leverage is a function used to calculate the variance of an estimation made by the model. The following equation relates the estimation variance with the leverage:

$$s_p{}^2 = s_{exp}{}^2 \, d(x)$$

Where $s_p{}^2$ is the variance of the estimation (value predicted by the model in a certain point x of the domain), $s_{exp}{}^2$ is the experimental variance and *d(x)* is the leverage in x. Therefore, leverage gives an indication on the precision that can be obtained on a prediction. Since the leverage for rotatable designs is symmetric, they can be considered balanced designs, as far as the precision of the prediction is concerned.

The most important and used experimental designs for modelling purposes are the three-level full factorial, the central composite (CCD), the Doehlert and the Box-Behnken [66,71,73]. All of them permit to build second order models and are balanced and symmetrical. Table 5 summarize their main properties, including the number of experiments involved.

Table 5: summary of the main characteristics of the response surface designs.

	Number of experiments (N)	**Orthogonality**	**Rotatability**
Three-level full factorial	$N= 3^k$	Yes	No
Central Composite	$N= 2^k + 2k + n_c$ *	Yes/No	Yes/No
Doehlert	$N= k^2 + k + n_c$	No	Only for k=2
Box-Behnken	$N= 2^k (k-1) + n_c$	Yes	Yes

*k is the number of factors and n_c is the number of the replicates of the central point.

The three-level full factorial is completely orthogonal and explores all the possible combination among the levels of the factors; it is characterized by a high number of experiments, since with 3 factors we already have 27 experiments, making this design not always feasible.

The central composite design can be seen as an extension of a two-level full factorial and explores the factors at 5 levels, coded as -α, -1, 0, +1, +α. It can be distinguished in faced centred or circumscribed, depending on the value of α. Fig. 8 represents geometrically the points of the domain investigated in both faced centred and circumscribed central composite designs for 3 factors.

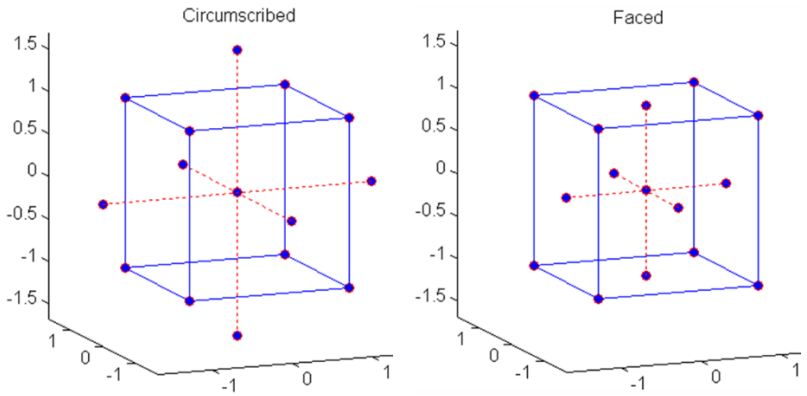


Figure 8: experimental points of a circumscribed and faced centred central composite design for 3 factors.

Central composite design can be orthogonal if $n_c = \alpha\sqrt{f} + 4 - f$ (where f is the number of factorial points) and rotatable if $\alpha = f^{1/4}$.

The Doehlert design explores the factors at different number of levels, depending on the number of factors. For example, if k=2, one factor is studied at 3 levels and one factor at 5 levels, if k=3, one factor is studied at 3 levels, one at 5 and one at 7. The coded levels are defined in the literature for a certain number of factors. This design is not orthogonal, while rotatable only for k=2, but has an interesting feature which makes it versatile and different from the others: it can be extended both in terms of factors and levels, adding some experiments to the already performed ones. This could be a great advantage when one is not 100% sure of which factors or domain to study. Fig. 9 shows the experimental points for a 2 factors design (a), as well as the possibility of extending the design with further levels (a) or factors (b).

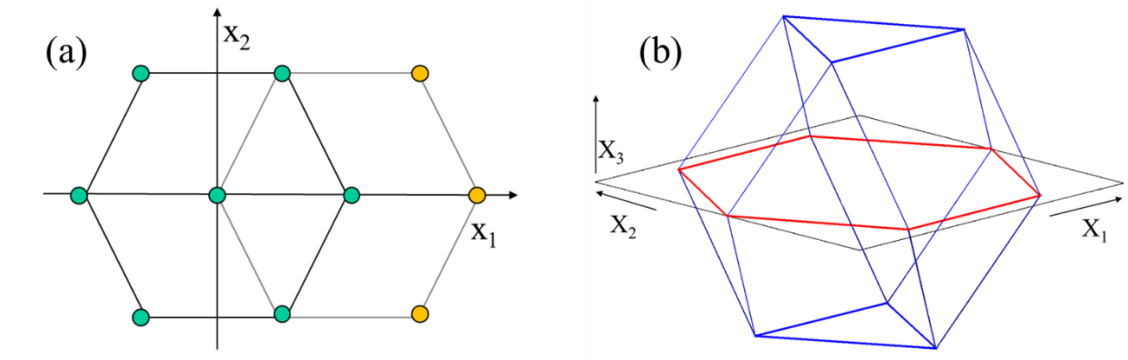


Figure 9: Experimental points for (a) a 2 factors Doehlert design (the yellow points being the levels extension) and (b) a 3 factors Doehlert design (in red the 2-factors design from which it was extended).

The Box Behnken design [74,75] was the one used during this research and was selected for its appreciable statistical properties. In fact, it is rotatable and defined as quasi-orthogonal: the

covariance among the coefficients is zero for most terms and very close to zero for the others. The factors are explored at 3 levels, coded as -1, 0 and +1 and the construction of the experimental matrix is easily obtained. For instance, the experimental matrix for a 3 factors design (12 points plus the central points) is composed by three blocks of 4 experiments in which one variable is fixed at the 0 level and the others have the sign alternation characteristic of a 2-level and 2 factors full factorial design (Table 6). Fig. 10 represents the experimental points derived from the described matrix.

Table 6: Experimental matrix for a Box-Behnken design with 3 factors.

exp	X_1	X_2	X_3
1	-1	-1	0
2	+1	-1	0
3	-1	+1	0
4	+1	+1	0
5	-1	0	-1
6	+1	0	-1
7	-1	0	+1
8	+1	0	+1
9	0	-1	-1
10	0	-1	+1
11	0	+1	-1
12	0	+1	+1
13	0	0	0

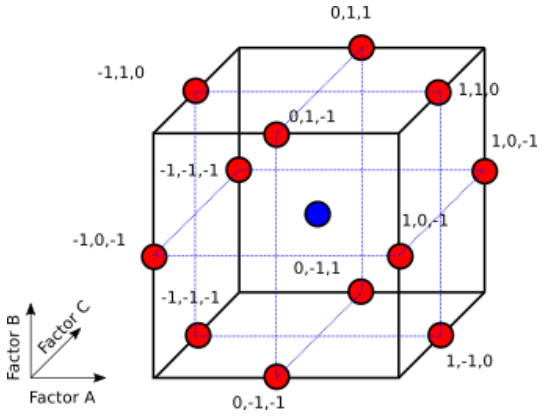


Figure 10: Experimental points of a Box-Behnken design with 3 factors.

Once the appropriate design and domain have been chosen and the experimental matrix built, randomization of the experiments must be accomplished, to mediate any effect due to time, or systematic drifts, which could be confused with the effect of a factor. For instance, if we perform all the experiments at level +1 of a factor in sequence, and during this time our instrumentation gives a better response, we will confuse the instrument performance with the effect of the factor. In this framework, it is also fundamental to perform at least 3 replicates of the central point, distributed

randomly in time, in order to have an actual estimation of the experimental variance over the entire time lapse of the experiments' performance.

Response surface designs are used to model the response as a function of the factors. Sometimes it is not straightforward to choose which response to model; for example, if the optimization of more than one response is necessary, some cumulative response could be necessary, in order to make data interpretation easier. If some responses are correlated, their summation or product could be considered, but a previous normalization of the data is commonly recommended in the case the responses are not of the same order of magnitude. Autoscaling is a simple data pre-treatment which is able to normalize the data, by the following formula:

$$R_{i\,Auto} = \frac{R_i - \hat{R}}{s}$$

Where $R_{i\,Auto}$ is the i-th autoscaled response, R_i is the response of the i-th esperiment, $\hat{R}$ is the average response of all the experiments and *s* their standard deviation. By autoscaling the responses, all of them will have the same weight in computing a sum or product. Another interesting data treatment which can be performed before model computation is Principal Component Analysis (PCA). This chemometric tool, which will be described in the next paragraph, is able to identify the important sources of variability of a set of data, discarding all information related to noise. If the responses are correlated, by PCA, we can select a new cumulative response instead of the starting ones and build one (or two) model(s) on a better set of data, theoretically free of non-significant variability.

Statistical evaluation and validation of the model

After considering all the described aspects, the experimental matrix, plus the column containing the responses (carefully pre-treated), is used to build the response surface. Multiple linear regression (MLR) is the technique exploited for model computation, which is a regression method extended to more than one variable. In order to determine the statistical significance of a model, a sufficient number of degrees of freedom should be present. The degrees of freedom of a model is equal to the total number of experiments minus the number of coefficients to compute. The higher the degrees of freedom, the better the estimation of experimental variability and therefore, significance of the coefficients of the model. Once obtained, a series of statistical evaluations of the model is made to establish its goodness and applicability, with the aid of mathematical and statistical software packages, as for example, the open source software "R". Normally, the evaluation is based on residual analysis and cross validation, considering the following parameters:

- **Explained variance (%)** – it is a fundamental value, which indicates how much of the variability of the data is explained by the model. It corresponds to R^2_{adj}, i.e. the determination

coefficient normalized to the degrees of freedom of the model. A model is considered to have an acceptable fitting if the % of explained variance is greater than 70%. Clearly, values greater than 90% are associated to excellent models.

- **Significance of coefficients –** it tells us the statistically significant variables and the level of confidence. Usually, a *p* value is associated to each coefficient, corresponding to the probability that the variation in the response given by that term is due to casual error. The smaller the *p* value, the higher the probability that the coefficient is significant. The following criteria determine coefficients significance:
  - ❖ $p<0.05$ (coefficient significant at the 95% confidence level)
  - ❖ $p<0.01$ (coefficient significant at the 99% confidence level)
  - ❖ $p<0.001$ (coefficient significant at the 99.9% confidence level)

  Experimental variance is used to assess significance, and an easy way to visualize it is by looking at a bar plot where coefficients are compared to the standard deviation of replicate experiments (Fig.11).

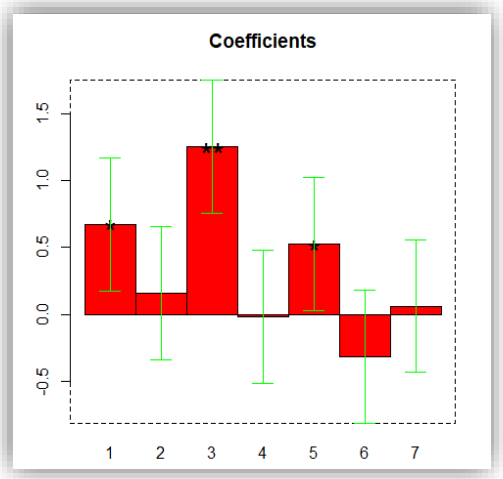


Figure 11: Example of bar plot representing the values of the coefficients of a model and the associated significance according to error bars, in green (experimental standard deviation).

- **Residuals** – they represent the difference between the experimental values and the fitted ones. They are usually plotted following the order in experiments performance; if the model is good and no lack of fit is present, a random and uniform distribution will be observed.
- **Standard deviation (of residuals)** – it is used for a comparison with the experimental standard deviation. An F-test can be performed with these two values in order to detect possible lack of fit (present if the two standard deviations are characterized by a big difference).

- **Explained variance (%) in Cross Validation –** it indicates the average percentage of variability explained by models computed by using the "leave-one.out" cross validation technique; it is a restrictive criterion, and only very stable and good models are characterized by high values of this parameter.

Analogously to the study of the described parameters, evaluations about the model goodness can be made using analysis of variance (ANOVA); although it could seem a more detailed and complete evaluation, the conclusions reached are normally very similar between the two "methods". Basically, ANOVA allows to establish the statistical significance of the model and its coefficients by performing a series of F-test on different sources of variation of the total data; these sources, associated to the computed model, are called "Sum of Squares" (SSQ). The following elements are necessary to define the SSQ used for the ANOVA of experimental design:

- n_{tot}= total number of the experiments (including central points and replicates)
- n_{obs}= number of observations (it does not consider replicates)
- n_r= number of replicates (of the central point or of each experiment, if performed in replicate)
- v= number of coefficients of the model
- y_{ij}= observed responses (where i represent a certain point in the experimental domain, while j represents the number of replicates)
- $\bar{y}_i$= average response for a certain point of the domain (if replicated)
- $\hat{y}_i$= fitted response for a certain point of the domain
- $\bar{y}$= average response

Table 7 summarizes the evaluated SSQ in ANOVA, with the mathematical formula as well as the related degrees of freedom.

Table 7: Definition of the sum of squares used for ANOVA and associated degrees of freedom.

Sum of Squares	Formula	Degrees of Freedom (DoF)
SSQ_{MOD} (model)	$\sum_{i=1}^{n_{tot}} (\hat{y}_i - \bar{y})^2$	$DoF_{MOD} = v - 1$
SSQ_{RES} (residuals)	$\sum_{i=1}^{n_{obs}} \sum_{j=1}^{n_r} (\hat{y}_i - y_{ij})^2$	$DoF_{RES} = n_{tot} - v$
SSQ_{LOF} (Lack of Fit)	$\sum_{i=1}^{n_{obs}} n_r (\hat{y}_i - \bar{y}_i)^2$	$DoF_{LOF} = n_{obs} - v$
SSQ_{PE} (Pure Error)	$\sum_{i=1}^{n_{obs}} \sum_{j=1}^{n_r} (\bar{y}_i - y_{ij})^2$	$DoF_{PE} = n_{tot} - n_{obs}$
SSQ_{TOT} (total)	$\sum_{i=1}^{n_{obs}} \sum_{j=1}^{n_r} (\bar{y} - y_{ij})^2$	$DoF_{TOT} = n_{tot} - 1$

By their definition, the sums of squares are characterized by the following equations:

$SSQ_{TOT}=SSQ_{MOD}+SSQ_{RES}$

$SSQ_{RES}=SSQ_{LOF}+SSQ_{PE}$

and the corresponding DoF follow the same additive rule.

For each sum of squares, a Mean Square (MS) is calculated, corresponding to the ratio among the SSQ and its DoF:

$$MS = \frac{SSQ}{DoF}$$

These MS, which are basically an estimation of the variances given by each source of variation, are then used to perform some F-tests; the following criteria establish the goodness of the model:

(1). Comparison between MS_{mod} and MS_{res}:

   a. if $F = \frac{MS_{mod}}{MS_{res}} > F_{crit}$ (p=0.05, DoF_{mod}, DoF_{res}), the model is significant with respect to the distribution of the residuals.

(2). Comparison between MS_{lof} and MS_{pe}:

   a. if $F = \frac{MS_{lof}}{MS_{pe}} < F_{crit}$ (p=0.05, DoF_{lof}, DoF_{pe}), the lack of fit is non-significant (in this case we want the lack of fit and pure error not to be statistically different)

(3). Evaluation of coefficients significance according to calculation of partial SSQs, obtained by eliminating one of the coefficients at a time. A term is considered significant if the percentage of explained variance of the model given by adding the coefficient increases in a significant way with respect to casual variability.

In other words, the first two F-test demonstrate that the majority of the variance of the data is explained by the model (1), and that the majority of the error is given by casual variability and not by the lack of fit, namely the inadequacy of the model (2) [73]. Usually, if these conditions are satisfied, also good R^2 and R^2 adjusted are found and the model can be considered a good approximation of the real data. After model computation and evaluation of its significance, it is common practice to report the model by writing only the significant coefficients, since, for prediction aims, the non-significant terms would only add noise to the estimated response.

Once obtained a satisfying model, an important step is its validation. We have to keep in mind that the validation of the model is not a demonstration of its trueness, but only a demonstration that it is not false. In fact, models obtained by response surface methodology are always approximations of the reality. Validation consists in performing a series of experiments, external with respect to the ones used to build the model, and in comparing the observed results with the ones predicted (fitted) by the model. It is advisable to perform these experiments in replicates, in order to estimate an error and verify the accordance of predicted and observed values on the basis of confidence intervals. It is common practice to validate a model in the centre of the experimental domain; this is because, for the majority of the experimental designs, in this point the leverage is minimum. Since the confidence interval for a prediction depends on the leverage value, having a minimum leverage means to have a smaller confidence interval in that point; therefore, if a model is validated in a point where the confidence interval is more restrictive, we can be confident that it will be validated also in points where the leverage is greater. Clearly, this statement is valid only if we assume homoscedasticity in the experimental domain, i.e. we have homogeneous experimental variance for all the points of the domain. The validation of the model in a point is accomplished if the predicted and observed values are not statistically different. We can perform a t-test where our responses are the following:

- Experimental value:

$$\bar{y}_{\exp(i)} \pm \frac{s_{exp}}{\sqrt{n_{exp}}} \quad , \quad DoF_{exp} = n_{exp} - 1$$

  Where $\bar{y}_{\exp(i)}$ is the average observed value in the i-th point of the experimental domain, s_{exp} is the experimental standard deviation and n_{exp} is the number of replicates.

- Prediction:

$$\hat{y}_i \pm \sqrt{d(x_i)} \cdot s_{expCP} \quad , \quad DoF_{pred} = n_R - 1$$

  Where $\hat{y}_i$ is the predicted value in the i-th point of the domain, s_{expCP} is the experimental standard deviation estimated by the replicates (n_R) of the central point during the experimental design and $d(x_i)$ is the leverage in the i-th point.

The t-test is performed in the classical way by computing the pooled variance and by using the critical t value at the 95% confidence level, selecting as degrees of freedom the sum of DoF_{exp} and DoF_{pred} $(n_{exp} + n_R - 2)$.

The final step in the use of experimental design and RSM is the identification of the optimal conditions for the process under study. From a mathematical point of view to find a maximum or minimum of the response curve, we need to calculate the partial derivatives of the function with respect to all variables, and to pose those derivatives equal to zero in a system; in this way we find the values of the variables for which we should have a minimum, maximum or saddle of the function. However, the points which satisfy the described mathematical system are not always the ones that are optimal for our goal. For example, if a maximum is located in a zone of the curve where the response is not stable, we could prefer another point of the response surface, as a compromise between high response and robustness. For this aim, the graphical visualization of the response model could be helpful and can be realized as the isoresponse plot (or contour plot) or the response surface. An example of the plots is reported in Fig. 12.

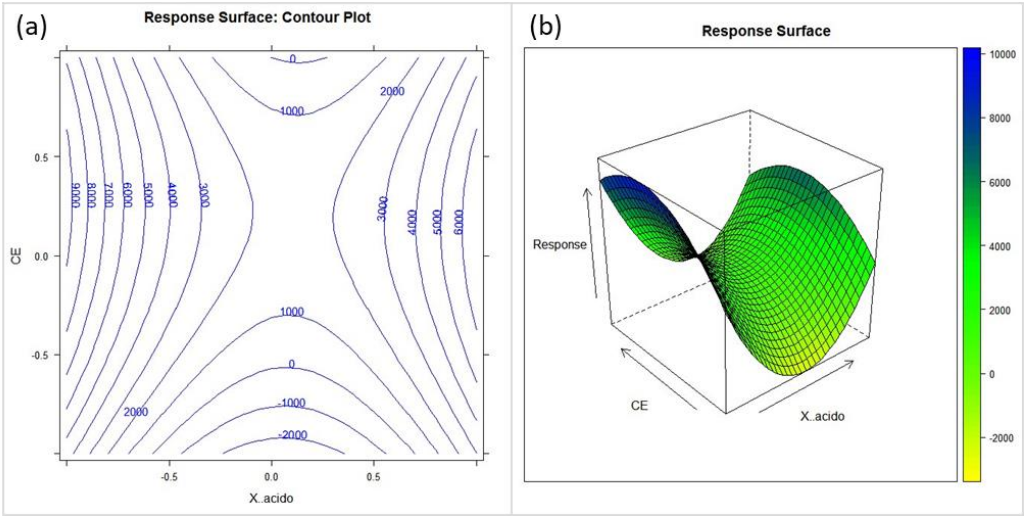


Figure 12: Example of an isoresponse plot (a) and a 3-D response surface (b) deriving from a quadratic model.

For both plots, only two variables at a time can be visualized. The variables which are not in the plot are fixed at a constant value, properly selected. The isoresponse plot consists of a two-dimensional plot in which the lines represent all the points having the same response, and the axes are two selected variables. This plot is useful to identify the conditions for which we have the desired response, whose value is commonly indicated above the lines. The response surface is a 3-dimensional curve, where we have two selected variables on the x and y axes, and the response on the z-axis. The 3-D representation usually makes it easier to understand the general trend of the model and locate the "sweet spot".

In some cases, the maximum (or minimum) of the curve could be outside the investigated domain; in such case, we could need the exploration of a different domain with the performance of a new set of experiments, or decide that a non-optimal point of the surface gives a satisfactory outcome for our purpose. Another aspect to consider is the optimization of multiple responses, which cannot be treated in a cumulative way. If more than one parameter should be optimized and the variables have different (if not opposite) effects on the different responses, it is not always easy to select the optimal conditions. A compromise is commonly reached by giving a certain priority to one (or more) response(s) with respect to others. A variety of strategies have been proposed to overcome cases when finding the compromise is not straightforward. One of the most used is based on the desirability function [71,76]. In this approach, the user assigns some weight to the single responses and then considers a product of the weighted values as a new response, the Global Desirability. The combinations for which this function is different from zero are considered points in which all responses have desirable values. A simpler approach is the use of the Pareto fronts [77,78]. First of all, a grid of candidate points that we consider characterized by acceptable responses is built by predicting the response for each point; these candidate points generate the Pareto front, which is represented in a plot of one response versus the other (or the other two, if we have 3 responses and a 3-D plot is used). Secondly, the "non-dominated" solutions are identified, that are those points for which all response values are at least as good as another point and at least one response is better than all the other points. If we have up to 3 responses, the Pareto fronts can be visualized, otherwise, a software can identify the non-dominated points, by selecting the proper constraints. Fig. 13 represents an example of Pareto front for two responses plotted against each other.

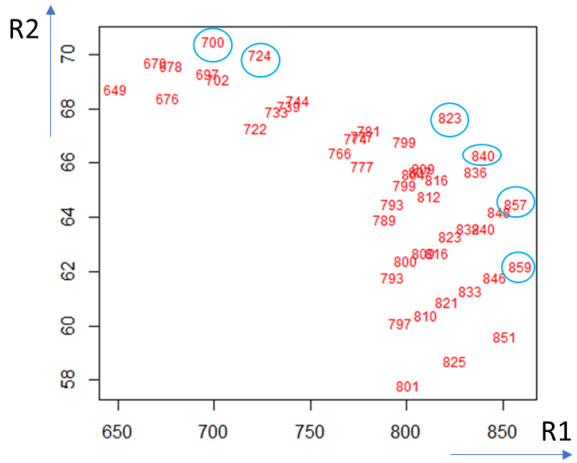


Figure 13: example of a Pareto Front for two responses; the points circled in blue are the non-dominated solutions.

Among the non-dominated points, the user can subjectively select the best option, on the bases of feasibility and other priority criteria.

Once the conditions for optimal response are identified, the corresponding experiment must be performed, and if the results are in accordance with what expected, the optimization process can be considered successfully concluded. The same steps performed for model validation can be used to evaluate the goodness of the prediction in the optimum and to compare the experimental and expected values.

We have to remember that the models obtained by experimental design are simple and local approximations of the reality, therefore an optimal response which does not perfectly fit the model could easily occur. Nevertheless, the goal of experimental design is to use a reliable and rational approach to find the optimum of a process, and by following the described steps, it is possible to achieve considerably better results than using a univariate approach.

2.3.2 Principal Component Analysis

Principal component analysis (PCA) is a chemometric tool, that is able to represent an original data set depending on a high number of variables, as a function of few new variables, the principal components (PC), which hopefully explain the most significant part of the variation of the data [79]. It is an unsupervised method (no previous knowledge about the data required), part of the field of exploratory data analysis.

The basic principle of PCA is to find correlation among the original real variables and hence construct new latent variables which are called principal components (PC). PCA decomposes an original data matrix (with as many rows as measured elements and as many columns as variables) as follows:

$$X_{(IJ)} = S_n \cdot L_n^T + E_{(IJ)}$$

where $X_{(IJ)}$ is the starting data matrix, n is the number of components, S_n is the *scores* vector ($S_n = [s_1 + s_2 + \cdots s_n]$), L_n is the *loadings* vector ($L_n = [l_1 + l_2 + \cdots l_n]$) and $E_{(IJ)}$ is the matrix of residuals, namely the part of information which is not explained by the model [80]. From the decomposition we can see that each initial variable and each element are represented as a function of the principal components: n loadings will be associated to each variable with respect to the n PCs; likewise, n scores will be associated to each entity. In other words, both variables and elements are represented by new coordinates in the space defined by the PCs. PCs are linear combinations of the starting variables; each PC sequentially explains a decreasing percentage of the total variance of the data and the PCs are orthogonal among each other [81]. The orthogonality implies that the percentage of variance explained by a PC is not explained by any other, while the decreasing percentage of variance explained is related to the fact that the first PCs explain the significant sources of variations,

while the last are generally associated to non-important information, i.e. noise. The following equation shows how the principal components are computed by the original variables:

$$PC_i = l_1x_1 + l_2x_2 + \cdots l_nx_n$$

where PC_i is the i-th principal component, x_i are the real variables (n variables) and l_i are the *loadings*, associated to the real variables. Therefore, the loadings express the weight that each starting variable possesses in defining the new component. As for the scores, they are the values that each element takes with respect to the new components, as if they were measured variables. Based on scores and loadings values, in PCA, we can visualize two types of scatter plots, the score plot and the loading plot, which show respectively the data points and variables, in a two or three-dimensional space defined by selected PCs. An example of both is shown in Fig.14.

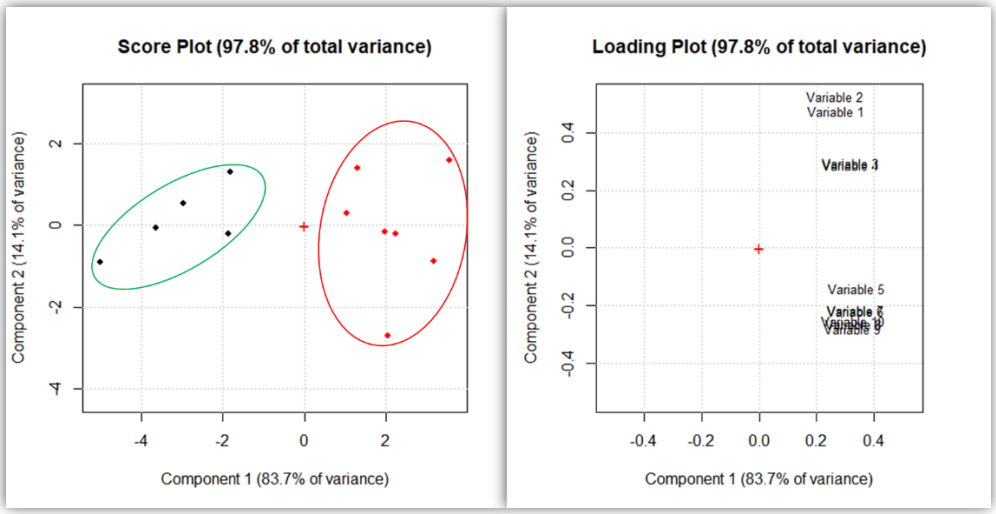


Figure 14: example of score and loading plot, obtained after performing PCA on a small data set.

The score plot allows to identify elements which are grouped, based on their similarities which emerge when PCs are computed; clearly, we must remember that they are similar as for the part of variability explained by the selected PCs [81]. As far as the loading plot is concerned, it permits to understand which variables are correlated and how much they contribute to the single PCs. If a variable has a high loading (both positive or negative) on a PC, that PC explains a significant part of variability given by the variable. Moreover, if two variables are close in the loading plot they are positively correlated, if they are opposite with respect to an axis they are anti-correlated, while if they are opposite with respect to the origin they do not have correlation. In the example proposed in Fig. 15 we can see that a very high percentage of variance is explained by only two PCs (97.8% of total variance), hence the visualization of the data in the PCs space is representative of the real variability; two groups of samples are identified by the score plot; the variables 1 to 4 and 5 to 10 are strongly correlated and all variables have a considerable contribute in the PCs construction, which can be easily deducted from the fact that over 97% of the variance is explained by the two principal

components. Another way to visualize the data and help the interpretation is the use of a *biplot*, which is the superposition of the score and the loading plot. By looking at a biplot we can understand the values taken by the samples with respect to the components, but also with respect to the starting variables: samples that are close or superposed with respect to an original variable will have a high value of this variable.

To summarize, PCA is used to gain the maximum information from the visualization of a data set depending on a high number of variables; it allows to identify patterns and similarities internal to the data and not visible by looking at them in a univariate way; finally, it puts the basis for classification supervised methods.

Chapter 3

Determination of phytoestrogens in soy-milk by GC-MS analysis after derivatization

3.1 Introduction

As stated in the first chapter, the most used technique for the quantification of phytoestrogens in food is liquid chromatography, usually coupled to mass spectrometry (LC-MS); a few examples of gas chromatography coupled to mass spectrometry (GC-MS) are reported for the analysis of phytoestrogens in food [55,56]. The reason why LC is usually preferred to GC is that for the latter method a derivatization step is necessary. In fact, phytoestrogens are slightly polar substances, characterized by low volatility, and this make them not suitable for direct GC analysis. Nevertheless, GC-MS remains a widespread technique, as well as less expensive in comparison to LC-MS, and it is interesting to test its possibilities in terms of sensitivity and specificity for phytoestrogens determination. To obtain reliable analyses, the derivatization procedure is a crucial step; in fact, it allows the GC analysis of polar compounds which otherwise would require excessively high temperature to elute from the column, with possible degradation. Polar compounds are usually characterized by -SH, -OH and -COOH functional groups which are able to form hydrogen bonds thus rendering the molecules not volatile. Modifying these groups by proper derivatization reactions

increases the volatility and enables the gas chromatographic analysis. The reagent used for this aim should fulfill some requirements:

- It should produce at least a 95% of derivative
- The derivative should be stable
- It should not cause structural modifications
- It should not give analytes loss
- The derivative should not strongly interact with the GC column

A range of reactions are used for derivatization, the most used being alkylation, acylation and sylilation. Alkylation provides the substitution of active hydrogen by an aliphatic group; it is achieved by the addition of dialkylacetals, diazoalkales, pentafluorobenzyl bromide and benzylbromide, among others. Acylation gives the replacement of a polar group with an acyl group, through an esterification reaction; common reagents are acetic anhydride, fluorinated anhydrides, N-Methyl-bis(trifluoroacetamide) and pentafluorobenzoyl chloride. Finally, silylation introduces a silyl group in place of active hydrogen, producing readily volatile compounds. Several compounds containing hydroxyl and amino groups (nonvolatile or unstable at the temperatures common reached during GC analysis) have been successfully analyzed in GC after silylation. Silylation occurs through nucleophilic attack (SN2), and the better the leaving group, the higher the reaction efficiency. A fundamental requirement for silylation reactions is the absence of water, which causes hydrolysis of the silyl esters formed; therefore, this derivatization should be performed in inert atmosphere (usually in gloveboxes with N_2 atmosphere). Moreover, the glassware should be inactivated with the silyl reagent prior to usage; in fact, the active sites present on the glass surface could subtract reagent to the analytes of interest, if not previously shielded. The most employed chemicals for this kind of derivatization process include hexamethyldisilzane, trimethylchlorosilane (TMCS), trimethylsilylimidazole, bistrimethylsilylacetamide, N,O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA), N-methyltrimethylsilyltrifluoroacetamide (MSTFA) and trimethylsilyldiethylamine, *N*-(tert-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MtBSTFA).

Derivatization of phytoestrogens is commonly achieved by silylation; the most used reagents are BSTFA, MSTFA and MtBSTFA, often in mixtures which include catalysts. BSTFA has been used in combination with 10% of TMCS to perform analysis of phytoestrogens in soy milk and wastewater [55]; derivatization with MSTFA has been applied for their determination in medicinal herbs [82] and estuarine water samples [83]; MtBSTFA with 1% of tert-butyldimethylchlorosilane (tBDMCS) has been selected as derivatization mix to analyze phytoestrogens in human urine [84]. In all these cases the derivatization procedure requires heating, incubation time (from 30 minutes to 4 hours) and different steps of evaporation and reconstitution of the solutions.

The aim of this work was to carefully study all the steps involved in the derivatization protocol to attain a new, easier and fast method for the analysis of phytoestrogens by GC-MS. The derivatization of the five phytoestrogens considered in this thesis (formononetin, biochanin A, daidzein, genistein and coumestrol) was optimized using different reagents and conditions; the multivariate approach of experimental design was used to rationally plan and perform the experiments. Moreover, a systematic study of the instrumental conditions for the analysis by GC coupled to an Ion Trap mass spectrometer was carried out: different methods were tested, changing the instrumental parameters (ion source temperature, tandem mass spectrometry settings, injection conditions) to enhance sensitivity and specificity of the technique. The developed method was validated and applied to the determination of the five phytoestrogens in some soy-based drinks from the Italian market.
The results presented in this chapter are object of a paper published in an international ISI journal (see Appendix: Publication 2).

3.2 Materials and methods

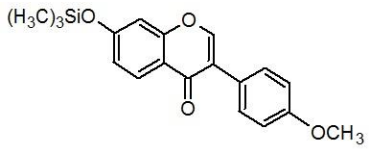
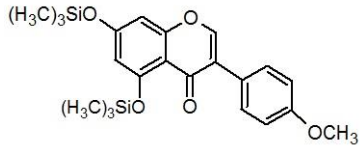
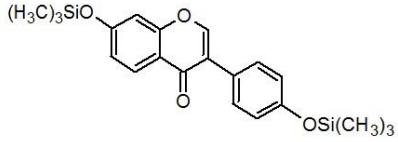
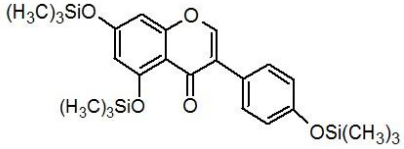
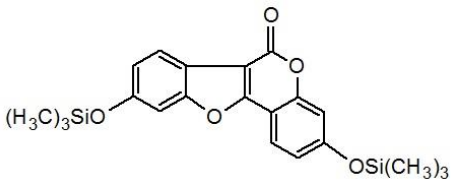
Chemicals and reagents

The phytoestrogens formononetin (FORM, >98%), biochanin A (BIOCH, >98%), daidzein (DAID, >98%), genistein (GEN, ≥98%) and coumestrol (COUM, ≥95%) were purchased from Sigma Aldrich (St. Louis, MO, USA). The derivatizing reagent N,O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA, 99.4 %) was obtained from Supelco (Bellefonte, PA, USA) while the derivatizing mixes N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) activated with ethanethiol and ammonium iodide, and N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MtBSTFA, >95%) with 1% of tert-butyldimethylchlorosilane (tBDMCS) were from Sigma Aldrich (St. Louis, MO, USA). The catalyst trimethylchlorosilane (TMCS, ≥99%) was obtained from Sigma Aldrich as well and was added to BSTFA to prepare a derivatizing mixture of BSTFA with 10% of TMCS.
Methanol (MeOH) was obtained from VWR Chemicals (Fontenay-sous-Bois, France), dichloromethane was from Lab Scan Ltd (Dublin, Ireland) and ethyl acetate and pyridine were purchased from Sigma Aldrich (Steinheim, Germany). All solvents were of chromatographic grade. Ultra-pure water was obtained from a Millipore Q-Gard system equipped with a Millipak 0,22 µm filter (Millipore, Watford, Hertfordshire, UK). Standard stock solutions were prepared in methanol at a concentration ranging from 180 to 1000 mg L^{-1} for the five phytoestrogens and kept at -20°C. The BSTFA:TMCS mixture was prepared under inert gas (N_2) and kept in a glass desiccator to prevent from hydrolyzation of the reagent.

Instrumentation and GC-MS/MS analysis

The analyses were performed using a Trace GC Ultra gas chromatograph coupled to an ITQ 1100 ion trap mass spectrometer, from Thermo Scientific (Rodano, MI, Italy), equipped with an AI-AS 1310 autosampler. The column used was a Thermo Scientific Trace Gold-SQC 30m x 0.25mm ID x 0.25µm (film thickness), with a composition of 95% methyl polysiloxane and 5% phenyl polysiloxane. The following method was the one optimized for trimethylsilyl (TMS) derivatives. The injection was of 1µL and a programmed temperature vaporizer (PTV) injector was chosen. The injector temperature program was the following: initial temperature of 45°C was held for 0.35 minutes during the evaporation phase; temperature increased to 280°C at 5°C sec^{-1} (held for 1 minute) during the transfer phase; in the final cleaning phase temperature increased to 350°C at 14.5°C sec^{-1} and was held for 10 minutes with a gas flow of 50 mL min^{-1}, to ensure the elimination of any carry-over effect. Carrier gas was helium at constant flow rate of 1.2 mL min^{-1} and the chromatographic separation was carried out by a 30 minutes run with the following oven program: initial temperature of 80°C (held for 5 minutes), followed by a first ramp to 230°C at 70°C min^{-1}, a second ramp to 300°C at 4°C min^{-1} and a final ramp to 310°C at 50°C min^{-1} (held for 5 minutes). The transfer line and ion source temperatures were set at 280°C and 250°C respectively. Positive electron ionization mode (EI+) was used with an electron energy of 70 eV. Tandem mass spectrometry was chosen to enhance sensitivity and MS/MS conditions were optimized for each phytoestrogen derivative. One precursor ion and two product ions were chosen for the identification and quantitation of the substances. Collision energies for the fragmentation were set at 1.5 V for all phytoestrogens except for daidzein which required a collision energy of 3 V. Selected ions for MS/MS detection of the TMS derivatives as well as their structures and retention times are shown in Table 8. Xcalibur software was used for data acquisition and processing.

Table 8: Retention times and selected ions for MS/MS detection of phytoestrogens' trimethylsilyl derivatives in the optimized GC-MS/MS method.

Compound	TMS derivative structure	Retention times (minutes)	MS/MS detection	
			Precursor ion	Product ions
Formononetin		16.01	340	325
Biochanin A		16.73	413	370, 398
Daidzein		17.29	398	355, 383
Genistein		17.64	471	327, 399
Coumestrol		19.35	412	369, 397

Derivatization procedures

The derivatization reagents tested for phytoestrogens were acetylating and silylating reagents.

The first test was performed trying an acetylation reaction, following the procedure developed in our laboratory for estrogens [85] with some modifications. The sample was prepared in duplicate and a procedural blank was obtained with the same procedure, starting from 500 µL of pure MeOH. Acetylation was tested by the following approach: 500 µL of standard solution of biochanin A at 2.5 mg L^{-1} were evaporated to dryness under a stream of N_2, 450 µL of a solution of acetic anhydride:pyridine (80:20, v/v) was added and the solution was vortexed and heated at 60 °C for 30 min in a thermostatic bath. After that, the obtained solution was dried under N_2 and reconstituted in 500 µL of ethyl acetate for the GC-MS analysis.

Three sylilation reagents were tested on standard solutions: MSTFA, MtBSTFA:tBDMCS (99:1, v/v) and BSTFA:TMCS (90:10, v/v). Each experiment was carried out in duplicate, always including a procedural blank, where the same procedure was applied to 200 µL of pure MeOH.

Derivatization with MSTFA (activated with ethanethiol and ammonium iodide) and MtBSTFA:tBDMCS (99:1, v/v), were performed following the procedure described respectively by Ribeiro et al. and Moors et al. [83,84]. Briefly, 200 µL of a phytoestrogen standard solution (DAID at 2.5 mg L^{-1}) were evaporated to dryness under a stream of N_2, then a volume of 200 µL of the derivatizing mix was added and the solution was vortexed for 1 minute and placed in a stove at 100°C for 1 hour (MSTFA derivatization) or in a thermostatic bath at 75°C for 30 minutes (MtBSTFA derivatization). After that, the solution was evaporated under a stream of N_2 and reconstituted in 200 µL of ethyl acetate or hexane before GC-MS analysis.

Finally, the derivatization procedure with BSTFA:TMCS (90:10, v/v), for the formation of the TMS derivatives of the phytoestrogens under study, was the one used by Ferrer et al. [55], with some modifications. The optimized protocol was the following: 200 µL of phytoestrogen standard solution or sample extract (in methanol) were evaporated to dryness under a stream of N_2 and 1 mL of dichloromethane was added and evaporated twice; this action allows the formation of an azeotrope between dichloromethane and water, thus ensuring the elimination of any trace of water from the sample, which could interfere with the silylation reaction and with the stability of the derivatives. 40 µL of pyridine and 160 µL of the BSTFA:TMCS mix were added to the dry residue; the solution was vortexed for 20 s and immediately analyzed by GC-MS/MS (injection port derivatization), using PTV injection. Some derivatization preliminary tests were performed following the approach usually reported in the literature, where the solution is heated before GC-MS analysis; in these experiments, pyridine and BSTFA:TMCS mix were added to the dry residue and the solution was put in a thermostatic bath at 70°C for 1 hour.

Experimental Design

For the optimization of the derivatization using BSTFA, the multivariate approach of experimental design was chosen, in order to take into account of possible interactions among the variables (factors) involved in the derivatization process. A simple full factorial design was performed, consisting in 2^k experiments, where k is the number of factors and 2 are the levels at which each factor is considered. Three experiments at the central point of the experimental domain were added to the design to estimate the variability associated to the performance of the experiments. In the derivatization procedure three are the variables involved: volume ratio between derivatization reagent and standard solution, temperature at which the derivatization takes place and incubation time of the solution.

The two levels of each variable have been chosen starting from literature data. Regarding temperature and time, an average of the values used for derivatization procedures in previous works [55,56,82–84,86] was selected as the center of the experimental domain and therefore used in the three repetitions of the central point experiment. These values were 60°C and 60 minutes respectively. Concerning the volume ratio between derivatization reagent and standard solution, the central value (1.5:1) was chosen to provide an excess of derivatization reagent moles in respect to the moles of phytoestrogens present in the standard solution at 2.5 mg L^{-1}. The minimum and maximum levels for each factor were fixed symmetrically to the central point values: the minimum was 40°C, 20 minutes and 1:1 ratio while the maximum was 80°C, 100 minutes and 2:1 ratio. Once selected the levels of each factor, the experimental matrix was built and the order of the experiments was randomized, as shown in Table 9.

Table 9: Full factorial experimental design for derivatization with BSTFA; experiments are shown in the order they were executed (random).

EXP	**X_1**	**X_2**	**X_3**
	T(°C)	**t (min)**	**V_{DER}/V_{STD}**
1	80	20	2
2	40	100	2
3	40	20	1
4	60	60	1.5
5	40	20	2
6	80	20	1
7	60	60	1.5
8	80	100	1
9	60	60	1.5
10	40	100	1
11	80	100	2

Each experiment was performed following the same steps already described for BSTFA derivatization, but changing the variables according to the levels set in the experiment plan. To take into account of instrumental fluctuations, after derivatization, an internal standard was added to each solution, using 10, 15 or 20 µL of dodecane at a concentration of 25 mg L^{-1}, depending on the total volume of the derivatizing reagent used in the experiments. The solutions were analyzed by GC-MS/MS.

Quantitative analysis

Calibration was performed preparing standard solutions in methanol at six different concentration levels: 8 µg L^{-1}, 20 µg L^{-1}, 50 µg L^{-1}, 100 µg L^{-1}, 250 µg L^{-1} and 500 µg L^{-1}. 200 µL of these standard solutions were derivatized following the optimized method and injected in triplicate. Intra-day and inter-day precision were evaluated for each level of the calibration curve; limit of detection (LOD) and limit of quantitation (LOQ) of the analytes were calculated considering the signal to noise ratio of 3 and 10 respectively. Since no reference materials are available for phytoestrogens, the recovery of the extraction procedure on soy drink samples was evaluated according to Matuszewski et al. [87]: two aliquots of the same soy milk were subjected to the extraction procedure; one aliquot (A) was spiked with a known amount of phytoestrogens before extraction and the other (B) was spiked after extraction. Recovery (RE) was then calculated for each phytoestrogen, through the following formula:

$$RE\ (\%) = 100 * \frac{PA_A}{PA_B}$$

Where PA_A and PA_B are the areas of the phytoestrogen derivative's peak obtained by analyzing extract A and B, respectively. The experiments were carried out in triplicate and the average recovery was calculated. Matrix effect was evaluated by comparing the calibration curve built with pure standards and the calibration curve obtained with the method of standard additions.

Sample preparation

Different soy-based drinks from the Italian market were purchased in a local grocery store and the developed method was used to assess the presence and concentration levels of the five phytoestrogens in these samples. Extraction and purification were simultaneously performed by Solid Phase Extraction (SPE) with Oasis HLB cartridges (60 mg, 3 mL, Waters, Dublin, Ireland). The extraction protocol was the following: conditioning of the cartridge with 1 mL of MeOH and 1 mL of water; loading of 1 mL of soy drink sample; washing with 2 mL of water; elution with 3 mL of MeOH. The eluates were dried under a stream of nitrogen and reconstituted with 1 mL of MeOH. Different concentration levels of the five phytoestrogens in soy-based drinks were expected, with a consistently higher content of GEN and DAID in comparison with the other phytoestrogens. For this reason, the methanol extracts were divided into two aliquots: one was directly derivatized and the other was subjected to a 200-fold dilution before derivatization. The two solutions were analyzed in triplicate.

3.3 Results and Discussion

Derivatization and instrumental conditions

The five phytoestrogens under study are slightly polar substances ($logK_{ow}$ in the range 2.5-3), not suitable for direct analysis by gas chromatography. Therefore, a derivatization step is needed, which requires a careful optimization of reagent type and reaction conditions. In fact, the derivatization procedure is a critical step to obtain derivatives that are suitable for gas chromatography and different reagents and modalities are used to perform the reaction. The most common derivatization compounds used for estrogens are acetylation and silylation reagents; therefore, these two classes have been tested on phytoestrogens, characterized by functional groups similar to those of estrogens. The acetylation reaction was assayed by adding acetic anhydride to a standard solution of biochanin A, following the protocol described in the previous "materials and method" section (Derivatization procedures). Unfortunately, acetylation was not successful; the extracted ion current (EIC) at the m/z value corresponding to the mass of the acetylated biochanin A did not reveal any peak. In fact, no difference was observed if compared with the chromatogram of the blank sample. This could indicate that the derivatization reaction didn't lead to the formation of the acetylated specie, maybe due to problems of steric hindrance. The failure of the acetylation reaction for phytoestrogens has been reported in the literature [84], therefore, no further tests were performed.

In the light of that, the focus was moved to silylating compounds, that are usually employed to derivatize molecules characterized by phenolic moieties and hydroxyl groups, such as phytoestrogens. Usually, derivatization is attained by using mixtures of different components which include a substance able to transfer silyl-functional groups, and one or more catalyst to promote the silylation reaction. The most commonly used are: BSTFA:TMCS (90:10, v/v) [55], MSTFA:NH_4I:2-mercaptoethanol (or ethanethiol) (1000:2:6, v/w/v) [83] and MtBSTFA:tBDMCS (99:1, v/v) [84]. The derivatization procedures lead to the formation of trimethylsilyl (TMS) derivatives (derivatization with BSTFA or MSTFA) and *tert*-butyldimethylsilyl (TBS) derivatives (derivatization with MtBSTFA), which are suitable for GC-MS analysis.

Some preliminary tests were performed on a single phytoestrogen standard solution (DAID) at a concentration of 2.5 mg L^{-1}; full scan acquisition mode was used for the GC-MS analysis. The three different derivatizing procedures with BSTFA, MSTFA and MtBSTFA, described in section 3.2, were tested on the DAID standard solution. All the three methods led to the detection of the daidzein silyl-ether peak in the chromatogram, with different intensity of the signals. As an example, Fig. 15 shows the results of the GC-MS analysis after BSTFA derivatization; the EIC (Extracted Ion Current) chromatogram of the DAID TMS derivative (m/z= 398), as well as its mass spectrum are shown.

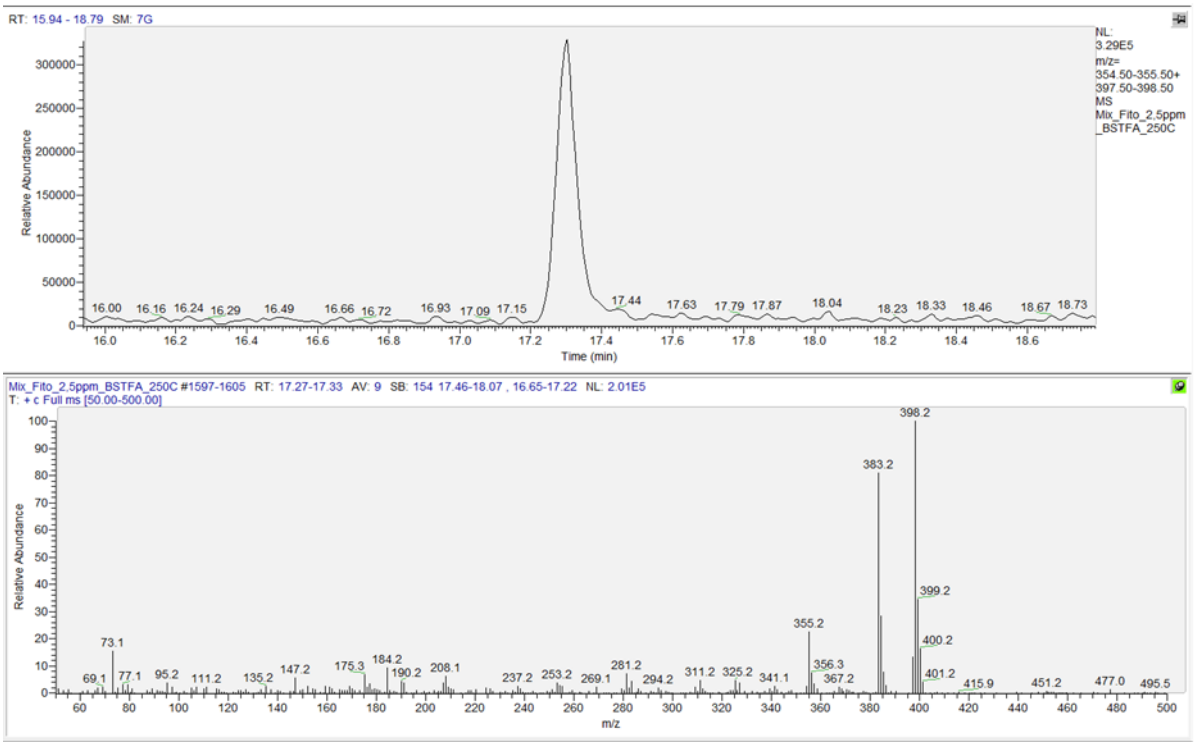


Figure 15: EIC chromatogram of the daidzein TMS derivative (extracted ion current at m/z=398) and mass spectrum of the peak.

The DAID derivative peak areas obtained from the GC-MS analysis were used to compare the efficiency of the three derivatizing compounds; the signal to noise ratio (S/N) was considered as an indicator of the sensitivity of the method. In this preliminary tests, the S/N values were quite low for all the experiments (10 <S/N< 76 for a standard solution at a concentration of 2.5 mg L^{-1}). Nevertheless, a significant difference between MSTFA and the other two derivatizing mixtures was found, with lower S/N values for MSTFA. Therefore, BSTFA and MtBSTFA derivatizing reagents were chosen to continue the experiments and were tested on the mix of all five phytoestrogens, using the same GC-MS method. Three out of five derivatives were detected (FORM, DAID and COUM derivatives) with poor sensitivity, while BIOCH and GEN derivatives peaks were absent. These problems could be due to an insufficient yield of the derivatization reaction, to low sensitivity of the methods or to a combination of both; therefore, various instrumental parameters were optimized, in order to enhance sensitivity, and an experimental design was performed to optimize the derivatization reaction.

First of all, GC-MS methods were compared to GC-MS/MS methods and, as expected, the MS/MS detection allowed a great increase of the sensitivity. The S/N value increased up to 20-fold using MS/MS detection compared to MS detection. Then, various instrumental conditions were studied: ion source temperature, tandem MS detection parameters and collision energies (CE) applied for the fragmentation of the ions.

Ion Source temperature

Ion source temperature was studied to verify its effect on the ionization of phytoestrogens and their subsequent detection. Two common operating temperatures (200°C and 250°C) were tested for the analysis of TMS and TBS derivatives. For TMS derivatives, best results in terms of S/N ratio were obtained at 250°C for all phytoestrogens. Regarding TBS derivatives, the three phytoestrogens exhibited a different behaviour at the two tested temperatures: while FORM showed an increase in sensitivity at 250°C, DAID and COUM gave better results at 200°C. For this reason, an intermediate temperature of 230°C was tested and resulted as a good compromise for the three analytes. The graphs in Fig. 16 summarize the obtained results.

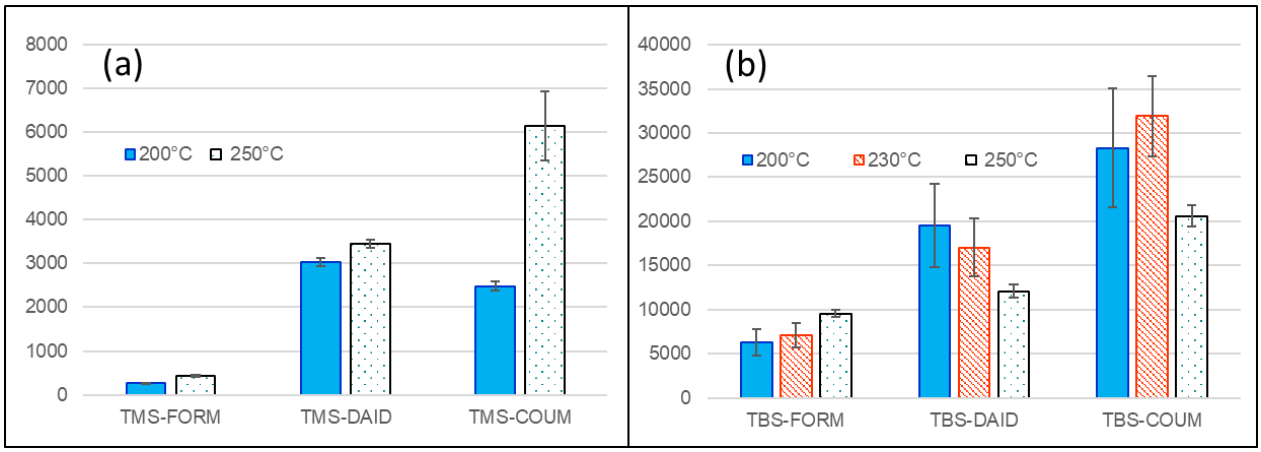


Figure 16 : Comparison of sensitivity obtained with different ion source temperatures: results shown as S/N values for the TMS derivatives (a) and TBS derivatives (b) signals of FORM, DAID and COUM (concentration of 2.5 mg L^{-1}).

Optimization of MS/MS detection

In order to increase the instrumental response during MS/MS experiments, a wide m/z range for product ions detection should be chosen to sum the signal intensities of all products and increase the peak areas. However, this approach could lead to a concomitant increase of chemical noise, proportional to the number of product ions detected [88]. Therefore, in this work, only the most abundant product ions plus the precursor ion were selected for the MS/MS detection, in order to minimize noise and thus enhance sensitivity. Moreover, fragments characterized by high m/z values were selected, to ensure high specificity. The monitored MS/MS transitions (selected precursor and product ions) for the TMS and TBS derivatives of the five phytoestrogens are shown in Table 10.

Table 10: selected precursor and product ions for TMS and TBS derivatives of the five phytoestrogens.

Analyte	TMS derivatives		TBS derivatives	
	Precursor ion	Product ions	Precursor ion	Product ions
FORM	340	325	382	325
DAID	398	355, 383	482	425
COUM	412	369, 397	496	383, 439
BIOCH	413	370, 398	512	455
GEN	471	327, 399	612	555

An important aspect to consider in MS/MS experiments is the choice of the ion isolation width. A wider m/z window should be chosen to include the isotopic cluster of an ion, if the signal intensities of the isotopic peaks are relevant. In our case, all analytes showed significant isotopic clusters for the chosen precursor and product ions, as we can see from mass spectrum of DAID TMS derivative, shown in Fig. 17 as an example.

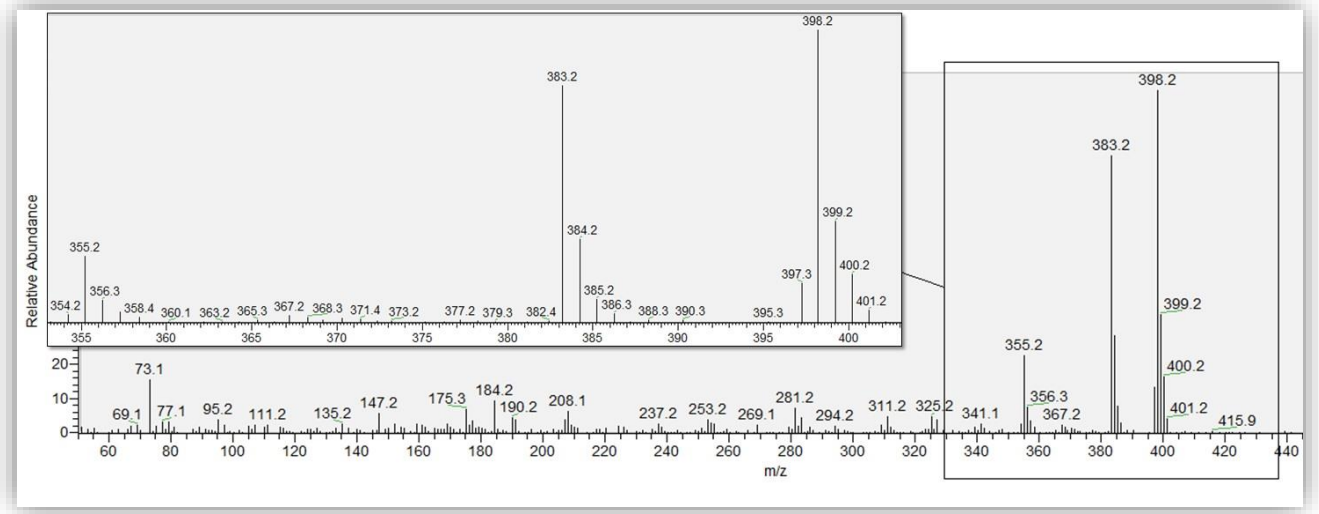


Figure 17- Mass spectrum of the daidzein TMS derivative: isotopic clusters of the selected precursor and product ions (m/z= 398, 383, 355).

Moreover, if the selected ions are characterized by low stability, a wider ion isolation width could be useful to ensure the trapping and detection of the wanted ions, as well as increasing intensity of the signals [89]. Nevertheless, the inclusion of more ions in the MS/MS detection necessarily leads to an increase of chemical noise. For this reason, we tested two different ion isolation width for precursor and product ions in performing MS/MS detection: m/z= x ± 1 and m/z= x ± 3 (where x is the m/z value of a selected ion). Even though the signal intensity was higher when detecting a wider m/z interval, a significant increase of S/N value (up to 8-fold) was registered when narrower ion isolation width was selected.

Thanks to the enhancement of sensitivity, BIOCH and GEN derivatives peaks were detected when BSTFA was used as derivatization reagent.

Collision energies

The fragmentation behaviour of the five phytoestrogens during the MS/MS experiments was also studied, changing the collision energy values. The tests were carried out with CE in the range of 1.5 - 5 V and in this case the absolute peak areas were compared, instead of the S/N ratios; in fact, in some cases, the noise signal tended towards zero. Optimum CE values were chosen to maximize the area of the phytoestrogens peaks in the MS/MS analysis. Furthermore, we ensured that the precursor ion peak maintained an intensity of about 10-20% compared to the intensity of the most abundant product ion; in fact, an excessive value of CE leads to the total fragmentation of the precursor ion and, therefore, could cause a fragmentation of the product ions as well, thus decreasing sensitivity. Optimum CE for analysis of TMS derivatives were 1.5 V for FORM, BIOCH, GEN and COUM and 3 V for DAID. Optimum CE for analysis of TBS derivatives were 2 V for DAID and 2.5 V for FORM and COUM.

After the careful study of the instrumental conditions, the best sensitivity was obtained for TBS derivatives. Nevertheless, BIOCH and GEN derivatives peaks were never detected when the derivatization was carried out with MtBSTFA. A reasonable explanation of this behaviour is a poor yield of the bimolecular nucleophilic substitution (SN2) reaction of MtBSTFA with these two analytes, due to steric hindrance causes. Problems of steric hindrance in silylation with MtBSTFA have been demonstrated for hydroxy-polycyclic aromatic hydrocarbons, sugars and phenols [90]. Among the five studied phytoestrogens, BIOCH and GEN are the only two with a carboxylic group adjacent to one of the phenolic group involved in the SN_2 reaction (shown in Fig. 18) and this could explain why they were not detected when silylation was performed with MtBSTFA.
For the described reason, BSTFA was chosen as the best derivatizing reagent and further experiments focused only on this derivatization procedure.

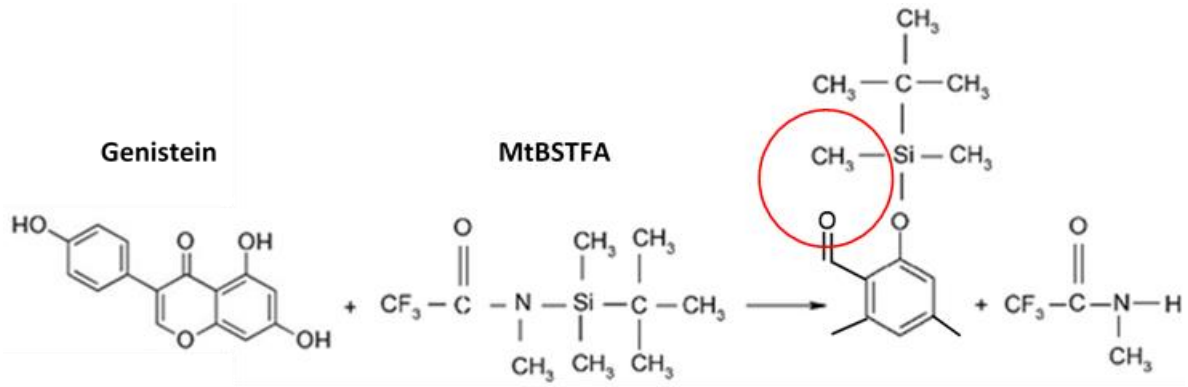


Figure 18: reaction of genistein with MtBSTFA: probable problem of steric hindrance given by the carboxyl group close to the phenolic group to be derivatized.

Results of the experimental design

The experimental design performed in this work was the full factorial, in which all the variables and first order interactions are taken into account to build the response model. This kind of design makes it possible to understand the influence of the variables and their interactions on the process under study [64]; by adding three experiments at the central point of the experimental domain, we obtained an estimation of the variability of the process and we could use this information to evaluate the significance of the effects of the variables involved. In our case, the factors considered were volume ratio between derivatization reagent and standard solution, temperature and incubation time of the solution. We wanted to verify the effects of these variables on the silylation reaction between phytoestrogens and BSTFA, in order to find the real optimal conditions for the process.

Once all the 11 experiments of the design were performed, the areas of the peaks obtained after GC-MS/MS analysis were normalized to the area of the internal standard and used to build the mathematical model of the response surface (equation 1):

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{123}x_1x_2x_3 \qquad \text{eq. (1)}$$

In the equation, Y is the normalized area of the phytoestrogen derivative peak (response), x_1, x_2 and x_3 are the values of the factors (temperature, time and volume ratios respectively) and b_i are the coefficients of each factor (or interaction of factors), indicating its effect on the response. Starting from the result of each experiment, the coefficients b_i were calculated, thus obtaining the response model. Then, the standard deviation associated to the results of the experiments was estimated through the repetition of the central point of the design; the values found, expressed as relative standard deviation (RSD), were between 20% and 26%. The significance of the coefficients is evaluated comparing it to a confidence interval, usually associated to a probability of 95% that the factor has an influence, greater than that given by casual variations. The confidence half-interval for 95% probability ($p_{0.05}$) is shown in eq.(2):

$$p_{0.05} = t \cdot \frac{s}{\sqrt{n}} \qquad \text{eq. (2)}$$

where *s* is the experimental standard deviation, *n* is the number of experiments of the full factorial design (2^3) and *t* is the t-student variable associated to the degrees of freedom which the experimental standard deviation is estimated with (N-1, with N=3 in our case). The computed coefficients and $p_{0.05}$ values for FORM, DAID and COUM are shown in Table 11; none of the coefficients was found to be significant, because all were lower in absolute value than $p_{0.05}$. In other words, a variation of any of the factor in the experimental domain caused a variation in the result lower than that given by casual fluctuations.

Table 11: coefficients of the response model calculated for FORM, DAID and COUM and their confidence half-interval (p0.05).

Coefficient	FORM	DAID	COUM
b_0	0.699	1.008	0.713
b_1	0.004	-0.009	-0.012
b_2	-0.010	-0.034	-0.002
b_3	-0.045	-0.069	-0.005
b_{12}	0.025	0.039	0.043
b_{13}	0.000	0.009	0.010
b_{23}	0.015	0.009	0.030
b_{123}	-0.030	-0.044	-0.035
$p_{0.05}$	0.26	0.39	0.34

This suggests that neither volume ratio nor time of incubation and temperature of heating before injection affect the derivatization reaction and its yield. The independence of the yield from the volume ratio was expected, since the minimum volume of derivatization reagent was already a large excess for the amount of phytoestrogens present in the standard solution. On the other hand, the non-significance of the other two factors (time of incubation and temperature of heating) suggests that the derivatization is instantaneous and probably occurs in the injector, where the solution is subjected to high temperature (280 °C) which promotes the phytoestrogens derivatization.

The occurrence of derivatization during the injection phase (or "injection port derivatization") has already been demonstrated for estrogens [91] and it is possible that the same happens for phytoestrogens.

Injection port derivatization and PTV injection

In order to verify if the injection port (inj-port) derivatization occurs for the considered phytoestrogens, two experiments were performed: in the first experiment, the phytoestrogens mix solution was subjected to GC-MS/MS analysis right after the addition of BSTFA (inj-port derivatization); in the second experiment, the derivatization was performed in thermostatic bath. At the same time, the application of a temperature program during the injection phase (PTV injection) was tested, to verify a potential benefit in the derivatization efficiency and reproducibility. Therefore, the solutions obtained by the two different derivatization procedures were analyzed by applying either a constant temperature (CT) injection at 280°C or a PTV injection. The inj-port derivatization was successful, leading to the detection of all phytoestrogens derivatives and therefore proving the independence of derivatization from the heating procedure applied before injection; the comparison between the two derivatization procedures (considering PTV injection mode) is shown in Fig. 19b.
Peak areas obtained from the inj-port derivatization were comparable or greater than those obtained with the classical derivatization mode (Table 12).

Table 12: Peak areas obtained from the GC-MS/MS analysis of phytoestrogens standard at 2.5 mg L^{-1}: comparison of injection port derivatization and derivatization in thermostatic bath with the two different injection modes.

	FORM ($*10^6$)	**DAID ($*10^6$)**	**COUM ($*10^6$)**	**BIOCH ($*10^6$)**	**GEN ($*10^6$)**
Thermostatic bath (CT injection)	1.04 ± 0.07	0.37 ± 0.03	2.1 ± 0.2	0.04 ± 0.01	0.11 ± 0.03
Thermostatic bath (PTV injection)	5.2 ± 0.2	0.71 ± 0.02	4.1 ± 0.1	1.2 ± 0.8	0.5 ± 0.3
Inj-port derivatization (CT injection)	1.0 ± 0.3	0.4 ± 0.1	2.1 ± 0.5	0.03 ± 0.02	0.06 ± 0.04
Inj-port derivatization (PTV injection)	5.04 ± 0.09	0.69 ± 0.03	3.96 ± 0.09	2.3 ± 0.8	0.9 ± 0.3

It is noteworthy that the greatest improvement given by inj-port derivatization was for BIOCH and GEN. This suggests that the derivatives of these two substances have low stability, and analyzing the solution right after the addition of the derivatizing reagent limits this problem.
Another interesting result was obtained with the PTV injection. It is known that a temperature program during the injection phase can be helpful for molecules with a relatively high molecular weight, in enhancing quantitative transfer to the chromatographic column as well as repeatability of the analyses [92]. In our case, the use of PTV led to a substantial improvement of sensitivity and repeatability. Peak areas increased from 2 to 5-fold if compared to CT injection; actually, BIOCH area in PTV injection was 77-fold higher than the area obtained with constant temperature injection (Table 12). The results are graphically displayed in Fig. 19a.

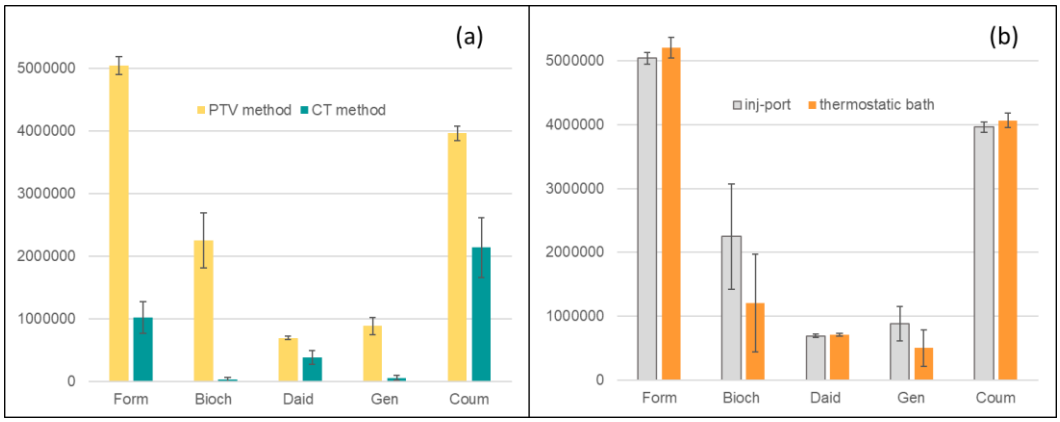


Figure 19 : comparison between PTV and constant temperature injection for the in-port derivatized standard (a). Comparison of inj-port derivatization and derivatization in thermostatic bath, both analyses performed with PTV injection (b);

Furthermore, the PTV injection led to considerable improvements in repeatability, with RSD% up to 6-fold lower for FORM, DAID and COUM; the comparison of RSD obtained with the two injection modes is shown in Table 13.

Table 13: Comparison of the repeatability (expressed as RSD%) of analyses with PTV and CT injection. Values represent the average RSD of inj-port derivatization and derivatization in thermostatic bath (both tested with the two injection modes).

	FORM	**DAID**	**COUM**	**BIOCH**	**GEN**
			(RSD)		
PTV injection	2.5%	3.4%	2.5%	50.2%	44.0%
CT injection	16.0%	18.5%	15.7%	72.9%	50.1%

Regrettably, BIOCH and GEN showed a very low precision in both cases, with slightly better results in PTV analyses. In fact, the chromatographic signals of BIOCH and GEN exhibited a considerable decrease from the first to the last replicate, suggesting that time and possible input of air and humidity after the first vial perforation by the auto-sampler needle, may hinder the derivatization reaction and cause the degradation of the compounds.

Method figures of merit

Summarizing up, the final method of analysis was the following: derivatization with BSTFA (as described in section “derivatization procedures”), performing the inj-port derivatization; PTV injection mode with temperature program from 45 to 280°C; GC-MS/MS analysis characterized by an ion source temperature of 250°C, ion isolation width of m/z= x ± 1, CE of 1.5 V for FORM, BIOCH, GEN and COUM and 3 V for DAID. The optimized method was validated in terms of linearity, detection and quantitation limits, precision, recovery and specificity. The obtained figures of merit for the five phytoestrogens are reported in Table 14; the method can be considered validated

for three of the five considered analytes, because, unfortunately, BIOCH and GEN showed rather poor figures of merit. This was not surprising, considering the problems encountered during the various optimization steps.

Table 14: Figures of merit of the developed method for the five phytoestrogens.

	R^2	Linearity (µg L^{-1})	LOD (µg L^{-1})	LOQ (µg L^{-1})	Recovery (%)	Intra-day Precision (RSD%)	Inter-day Precision (RSD%)
FORM	0.9984	8-500	1.1	3.6	72	4.7	12.4
DAID	0.9978	8-500	1.8	5.9	74	6.1	13.5
COUM	0.9996	8-500	0.1	0.3	53	4.8	12.1
BIOCH	0.9650	50-250	17.7	59.2	47	54.9	-
GEN	0.9750	50-250	3.9	13.1	50	50.6	-

The linearity was verified in the range of 8-500 µg L^{-1}, achieving good determination coefficient (R^2) for FORM, DAID and COUM (>0.99). Intra-day and inter-day precision, expressed as relative standard deviation (RSD), were in the range 4.7-6.1% and 12.1-13.5% respectively. The calibration curves for BIOCH and GEN showed low R^2 values in a reduced concentration range; these two analytes presented also very high RSD values in intra-day replicates, while it was not possible to evaluate the inter-day precision. For this reason, the developed method can only give an estimation of the BIOCH and GEN content in the soy milk samples. Recovery was acceptable, in the range 47-74%, with the lowest values found for BIOCH and GEN. The optimized method provided a very good sensitivity, with LODs and LOQs in the range of 0.1-17 µg L^{-1} and 0.3-59 µg L^{-1} respectively. These values are comparable or lower than other GC-MS methods reported in the literature for phytoestrogens [55,82,84].

The matrix effect was evaluated, as well as specificity of the method. A calibration curve was built for FORM, DAID and COUM with the standard addition method, i.e. by adding known amounts of phytoestrogens to the matrix (methanol extract of soy milk) and the calibration curves obtained showed angular coefficients comparable to those obtained with the external calibration; ratio between the two angular coefficients were in the range 0.99-1.1, indicating that matrix effect was negligible.

To verify the specificity of the method, the retention times and mass spectrum of the five phytoestrogens in the standard solutions and in the soy milk extracts were compared. Thanks to the MS/MS detection we obtained high specificity of the method. In fact, the same ratio between the selected ions peaks was found in the standard solutions and in the real samples; this suggests that no

interferences at the retention times of the phytoestrogens derivatives occurred, since any coeluted compound would change the mass spectrum associated to the chromatographic peak.

Application to soy milk samples

The developed method was applied to the analysis of three commercial soy milks available on the Italian market. In soy-based drinks GEN and DAID are usually found in the concentration range of tens of mg L^{-1} [55,93,94] while COUM is at much lower concentration; Kuhnle et al [44] indicated that COUM was under their LOD in soy milk samples (<10 µg L^{-1}). Often the concentration in soy milk is given as a sum of all isoflavones (including FORM, BIOCH, DAID and GEN), at levels of 50-60 mg L^{-1} [43,46], without specifying the contribution of the specific compound.

In this work, due to the different levels of concentration expected, two samples for each soy milk were prepared: the first sample was analyzed without any dilution, to measure the less abundant phytoestrogens. A 200-fold dilution was applied to the second sample, to quantify the most concentrated analytes, which would otherwise fall out of the linearity range. Final resulting concentrations for all phytoestrogens are shown in Table 15.

Table 15: concentrations of the five phytoestrogens in the three soy milk samples and associated standard deviations.

	Soy milk 1 (mg L^{-1})	**Soy milk 2 (mg L^{-1})**	**Soy milk 3 (mg L^{-1})**
FORM	<LOD	0.033 ± 0.003	0.024 ± 0.007
DAID	4.1 ± 0.9	7 ± 1	10.4 ± 0.7
COUM	<LOD	0.033 ± 0.002	0.028 ± 0.004
BIOCH	0.07 ± 0.04	0.2 ± 0.1	0.4± 0.2
GEN	16 ± 8	10 ± 6	16 ± 8

As extensively discussed in previous paragraphs, the results for BIOCH and GEN must be considered an estimation of their concentration; this is clearly highlighted by the extremely high standard deviation values. Concentration levels of the five phytoestrogens in the analyzed samples show some differences. As expected, GEN and DAID were present at rather high concentrations, ranging from 4 to 16 mg L^{-1}; on the other hand, FORM and COUM were the less abundant analytes and were under the method limit of detection in soy milk number 1. There was no evident correlation between each compound and the total phytoestrogen content in the analyzed samples: a higher concentration of one or more analytes did not correspond to a higher total amount of phytoestrogens.

The only relevant information provided in the label of these commercial soy milks regards the soy percentage: samples 1, 2 and 3 contain 5.9%, 8 % and 6.7 % of soy respectively. Again, no correlation

seemed to exist between the percentage of soy present in the samples and the measured amount of the five phytoestrogens; we hypothesize that both the variety of soy employed and the differences in the manufacturing process of each commercial product may have an influence on the final concentration level. A concluding remark regards the soy milk number 2 that, differently from the others, reported in label the indication “organic”; nevertheless, this feature did not correspond to any particular trend in the concentration of the phytoestrogens under study.

3.4 Conclusions

The developed GC-MS method, based on derivatization with BSTFA, was suitable for the determination of phytoestrogens in commercial soy milks; thanks to the careful study of the instrumental parameters involved, very good sensitivity and specificity were reached.

All the five considered compounds were detected at not negligible concentrations, in particular GEN and DAID were measured at the ppm level. Regrettably, the quantitation of BIOCH and GEN was affected by quite low precision. Good figures of merit were achieved for the other phytoestrogens. The careful optimization of the derivatization step by a multivariate approach clearly indicated that the incubation time and the heating temperature are not significant parameters, suggesting the possibility to perform the phytoestrogen derivatization directly in the injector, with a noticeable saving of the analysis time. Besides, the PTV injection using a thermal gradient led to considerable improvements in sensitivity, in particular for BIOCH, that showed an increase of the signal of about two orders of magnitude. This injection mode also increased precision, especially for FORM, DAID and COUM, giving 6-fold lower standard deviation in comparison with constant temperature injection. The proposed method is fast, simple and fully applicable for the determination of FORM, DAID and COUM in soy drink samples; to reach higher precision for BIOCH and GEN, other methods, such as HPLC-MS, must be considered.

Chapter 4:

Comparison of GC-MS and LC-MS for the analysis of phytoestrogens in soy milk

4.1 Introduction

Despite phytoestrogens are not suitable for direct GC analysis, thanks to the optimized derivatization step, it was possible to perform a rapid and simple procedure to determine their concentration in soy milk by GC-MS. However, although gas chromatography can be employed, derivatization could be tricky and impractical, making direct analysis preferable, when possible. In fact, a large employment of LC based analytical methods is reported in the literature for the analysis of phytoestrogens. Liquid chromatography was used to determine phytoestrogens naturally present in food and plants [6,44,46,95], as well as in environmental samples, where they are considered contaminants [96–100]. Usually, when analysing the most abundant phytoestrogens in natural sources (vegetables), the relatively high expected concentration levels makes it possible to utilize detectors such as UV or DAD, whose LODs are usually in the range of hundreds of μg L^{-1}. On the other hand, phytoestrogens contamination of water and soil normally does not exceed the low ng L^{-1} level, requiring either high pre-concentration factors (e.g. using large loading volumes in solid phase extraction) or more sensitive detectors coupled to the liquid chromatograph, such as mass spectrometers. The use of

sensitive detectors is clearly preferred, since exaggerate pre-concentration could lead to important matrix effects due to the possible pre-concentration of interferent species joint with the analytes of interest. The analytes considered during this thesis include compounds which are present in soy-based food at high concentrations (genistein and daidzein), but also others which are at the trace level. Therefore, we decided to develop a HPLC-MS/MS method for the determination of the analytes of interest in the soy milk matrix and to compare it with the GC-MS/MS one. The figures of merit of the two analytical methods and the results of soy milk analysis were compared.

4.2 Materials and methods

Chemicals

The phytoestrogens standards (DAID, GEN, FORM, BIOCH and COUM) were the same used in the previous part of the work, purchased from Sigma Aldrich (St. Louis, MO, USA). Methanol (MeOH) and acetonitrile (ACN) were from VWR Chemicals (Fontenay-sous-Bois, France), while formic acid (98%) was obtained from Sigma Aldrich (Steinheim, Germany). All solvents were of chromatographic grade.

Ultra pure water was obtained from a Millipore Q-Gard system equipped with a Millipak 0.22 µm filter (Millipore, Watford, Hertfordshire, UK).

Instrumentation and LC-MS analysis

The analyses were performed using an Agilent 1200 SL high performance liquid chromatograph equipped with a binary pump, an online vacuum degasser, an ALS automatic sampler and a thermostatted column compartment (Agilent Technologies, Santa Clara, CA, USA). The coupling was with an Agilent 6430 triple quadrupole (QqQ) mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) with an ESI ion source. The chromatographic column was a reverse phase C18 column (Zorbax SB-C18) of 50 mm (length), particles diameter of 1.8 µm and internal diameter (ID) of 2.1 mm by Agilent technologies, Santa Clara, CA, USA). After testing different chromatographic conditions, the best separation was achieved with a run of 13 min. The injection was of 10 µL, the column was kept at 25 °C, the flow was of 0.25 mL min^{-1} and the mobile phases were H_2O with 0.01% (v/v) of formic acid (phase A) and acetonitrile (phase B). The following gradient was applied: initial 90% of phase A (0.01% formic acid in water), hold for 1.5 minutes, increase of phase B (ACN) to 25% at minute 2.5, hold for 1 minute, increase of phase B to 80% at minute 8, hold for 3 minutes, return to the initial conditions in 1 minute, hold for 1 minute. As far as mass spectrometry is concerned, ESI positive ionization mode was selected for the five analytes, based on literature data; ESI conditions were characterized by a drying gas flow (N_2) of 10 L min^{-1}, drying gas temperature of

350 °C, capillary potential of +3000 V and nebulizer pressure of 35 psi. Mass calibration for MS experiments was performed by infusion of ESI-L low concentration Tuning Mix (G1969-85000 by Agilent Technologies, Santa Clara, CA, USA).

The tandem MS detection was in multiple reaction monitoring (MRM) mode; two or three transitions were chosen for each compound and the MS settings were optimized (dwell time, fragmentor voltage, collision energy, cell accelerator voltage). The Agilent MassHunter workstation software (version B.03.01) was used for data acquisition and processing and the Agilent MassHunter Optimizer software (version B.01.04) was used for the MRM optimization. Product ions spectra were acquired as well, to confirm the results obtained by the Optimizer tests; the scan window of the second quadrupole (MS2) was set in the range 60-285 m/z, and collision energies of 30 and 40 V were selected to acquire the product ions spectra.

Sample preparation and analysis

The soy milk samples were pre-treated following the SPE procedure already described in chapter 3 (materials and method section). The 3 mL MeOH eluate obtained from the SPE, was dried under N_2 and reconstituted in 1 mL of the same solvent. An aliquot of this solution was subjected to a 200-fold dilution in MeOH, for the quantitation of the most concentrated analytes, namely GEN and DAID; another aliquot was directly analyzed by LC-MS/MS. During a set of analyses two blanks (pure MeOH) were analyzed after each sample, in order to verify the absence of any carryover effect.

4.3 Results and Discussion

Method development

In order to optimize a HPLC-MS/MS method for phytoestrogens determination in soy milk, both chromatographic separation and MS detection (in multiple reaction monitoring mode) were considered. Chromatographic separation was optimized first, by setting the MRM parameters for the five phytoestrogens at values found in the literature. Once the best chromatographic conditions were found, method sensitivity was improved by optimizing the MS detection.

The column employed for the development of the HPLC method was characterized by a classical stationary phase for reverse-phase chromatography, namely a C18 phase. This choice was made based on previous works found in the literature for the separation of phytoestrogens [46,97,101,102]. Different separation conditions were tested, taking into consideration several variables, such as mobile phases, flow and applied gradient. The first test was performed considering the gradient used and the retention times observed in a work by Delgado-Zamareño et al [48]. Their gradient was

characterized by a very high percentage of water (with 0.01% of formic acid) in the initial stage, and a slow increase of organic phase (acetonitrile, ACN); however, the isoflavones' retention times were in the final part of the chromatographic run, which was of 12 minutes. Therefore, we decided to test a simpler run of 10 minutes, using a flow of 0.2 mL min^{-1}, a temperature of 25°C and the following gradient: initial 60% of phase A (0.01% formic acid in water), hold for 1 minute, increase of phase B (0.01% formic acid in ACN) to 80% at minute 3, hold for 4 minutes, return to the initial conditions in 1 minute, hold for 2 minutes (for column re-equilibration). Figure 20 shows the chromatogram obtained after analysis of a 50 µg L^{-1} phytoestrogens solution with the described separation conditions. Unfortunately, very bad separation was achieved and, more importantly, the peak shape was unacceptable; in fact, all compounds were characterized by broad peak tailing, which contributed to the poor resolution of the peaks.

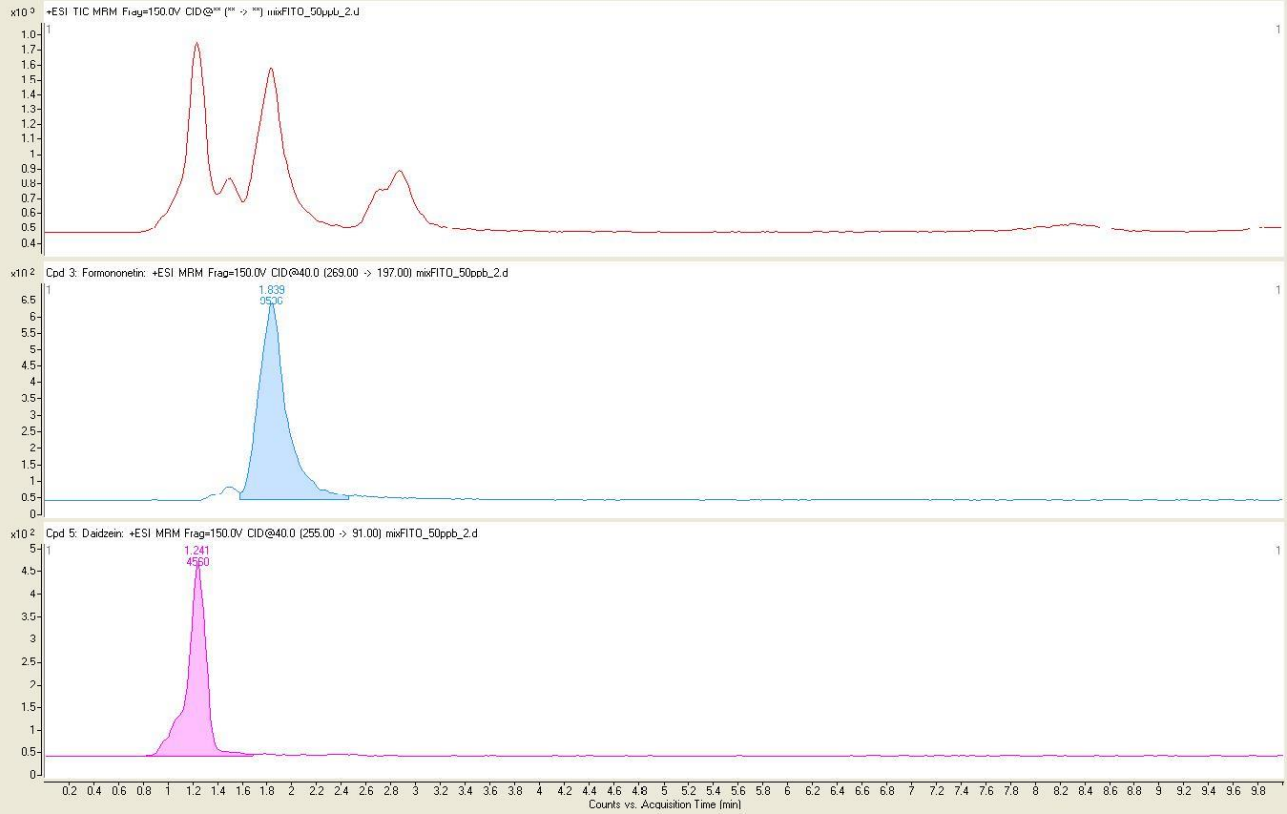


Figure 20: Chromatogram obtained with the first tested gradient, highlighting the encountered problem of peak broadening and tailing. The EIC of the MRM transitions (literature MS parameters) of FORM and DAID are shown in blue and pink respectively.

Peak tailing is a phenomenon which can be ascribed to problems in the mass transfer from the stationary to the mobile phase, such as slow desorption rates [103]. To overcome this problem, as well as to obtain acceptable separation, different aspects were considered: phases pH, gradient used, type of mobile phase and analysis temperature. Firstly, the chromatographic gradient was slowed down by applying the following time program: initial 60% of phase A (0.01% formic acid in water), hold for 1 minute, increase of phase B (0.01% formic acid in ACN) to 70% at minute 8, return to the

initial conditions in 1 minute, hold for 2 minutes. A better separation was attained, but no improvement in peak shape was observed. Therefore, some trials were carried out changing the amount of formic acid added to the mobile phases. Two opposite conditions were tested: in the first one the percentage of formic acid was increased to 0.1% in both mobile phases; in the second one neutral phases were used for the chromatographic separation. No improvement was detected in neither cases, suggesting that acidity of the mobile phase was not a critical aspect in determining peak shape. Hence, MeOH, in alternative to ACN as organic phase (phase B), was tested, repeating the same assessments already executed (different acidity of the solvents). Results were deceiving, since MeOH led to even worse peak broadening, in all pH conditions. Accordingly, we decided to use water with 0.01% formic acid as phase A and pure ACN as phase B, which were the solvents proposed by Delgado-Zamareño et al. The effect of temperature (up to 40°C) was studied as well, but once more, no significant improvement was observed at different column temperatures; therefore 25°C were set as analysis temperature.

At this stage, we focused on the optimization of the chromatographic gradient and flow. After several attempts, the flow was set at 0.25 mL min^{-1} and this final gradient allowed to obtain peak separation and satisfactory peak shape: initial 90% of phase A (0.01% formic acid in water), hold for 1.5 minutes, increase of phase B (ACN) to 25% at minute 2.5, hold for 1 minute, increase of phase B to 80% at minute 8, hold for 3 minutes, return to the initial conditions in 1 minute, hold for 1 minute. An additional post time of 6 minutes was used to re-equilibrate the column at the initial conditions.

The accurate study of all chromatographic variables, led to the conclusion that a high percentage of aqueous phase is required at the initial stage of the chromatographic run to obtain sharp peaks. We suppose that a high water percentage facilitates the binding of the molecules to the stationary phase, which otherwise tend to widely diffuse, causing peak broadening and tailing. Indeed, water tends to prefer forming water-water interactions, thus assisting in the transfer of the compounds to the stationary phase [104]. A chromatogram obtained with the final method is shown in Fig. 21.

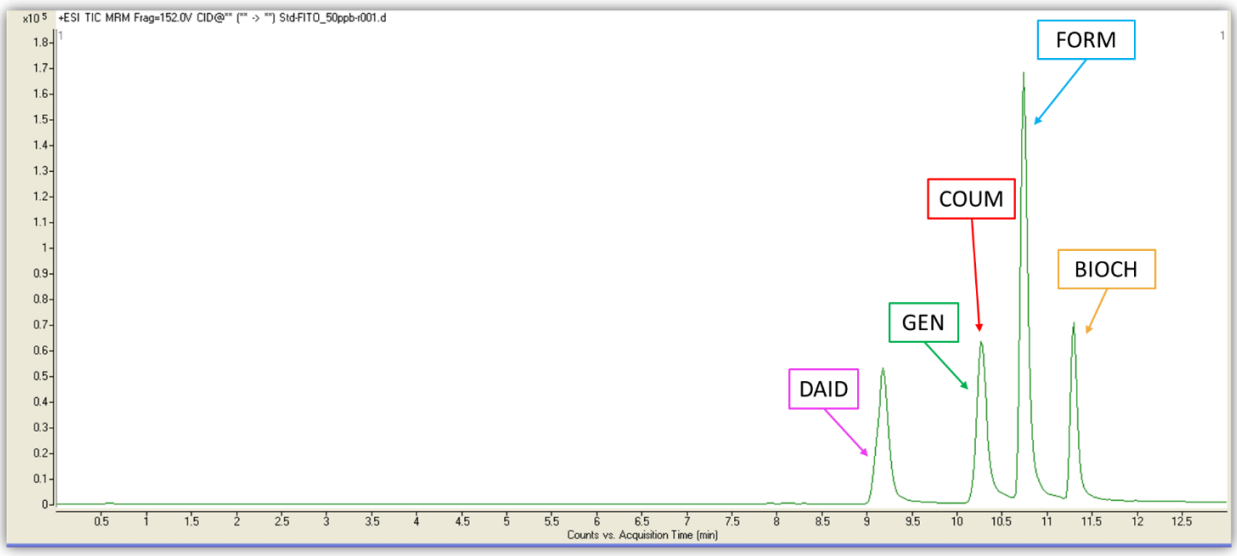


Figure 21: chromatogram obtained by the analysis of a phytoestrogen mix at 50 µg L-1 with the final separation gradient.

As can be seen, only 4 peaks were resolved, since coelution of GEN and COUM could not be avoided. Nevertheless, thanks to their different MRM transitions, chromatographic separation was not mandatory. In fact, by extracting the current of each MRM transition, the two peaks can be visualized separately. This great advantage of the MRM mode was one of the main reason why it was chosen in the MS/MS detection. When using a triple quadrupole analyzer, MRM mode is considered the best option to reach high specificity and maximum sensitivity. However, a careful study of the best transitions, as well as the appropriate values of several MS parameters, is necessary for attaining optimal sensitivity and selectivity. Two or three transitions were identified for each compound using the Optimizer software and the results were confirmed by literature data and product ions spectra acquisitions. The software tests and optimizes the MRM acquisition parameters for the selected compounds, after some chromatographic runs, without the need of direct infusion in the triple quad spectrometer. In particular, five injections of single standard solutions (concentration of 500 µg L^{-1}) were performed, and the software sequentially selected the best values of the following MS parameters: precursor ions, fragmentor voltages, product ions and collision energy (CE) values. The Optimizer's runs were performed after specifying the mass of each molecule and the ranges for the above-mentioned MS parameters. Since positive ionization mode was selected, the most abundant precursor ions for all compounds were the protonated molecular ions $[M+H]^+$. The lower mass cut off for the product ions was set at m/z=30 and the ranges for the MS parameters were 40-180 V for the fragmentor voltage and 0-60 V for the collision energy (CE). After the injections, two or more MRM transitions were identified for each compound, with associated abundance data. Among these transitions, the most abundant one was selected as the quantifier transition, while the one or two others were used for confirmatory purposes (qualifier transitions).

The results were mainly in accordance with literature data and were further verified with the acquisition of product ions spectra at selected CE values and specific m/z ranges. We chose CE of 30 and 40 V, which were intermediate values for all selected transitions, and verified which ions were the most abundant at the two different energy levels. The product ions spectrum of DAID at CE of 30 V and 40 V is shown in Fig. 22 as an example.

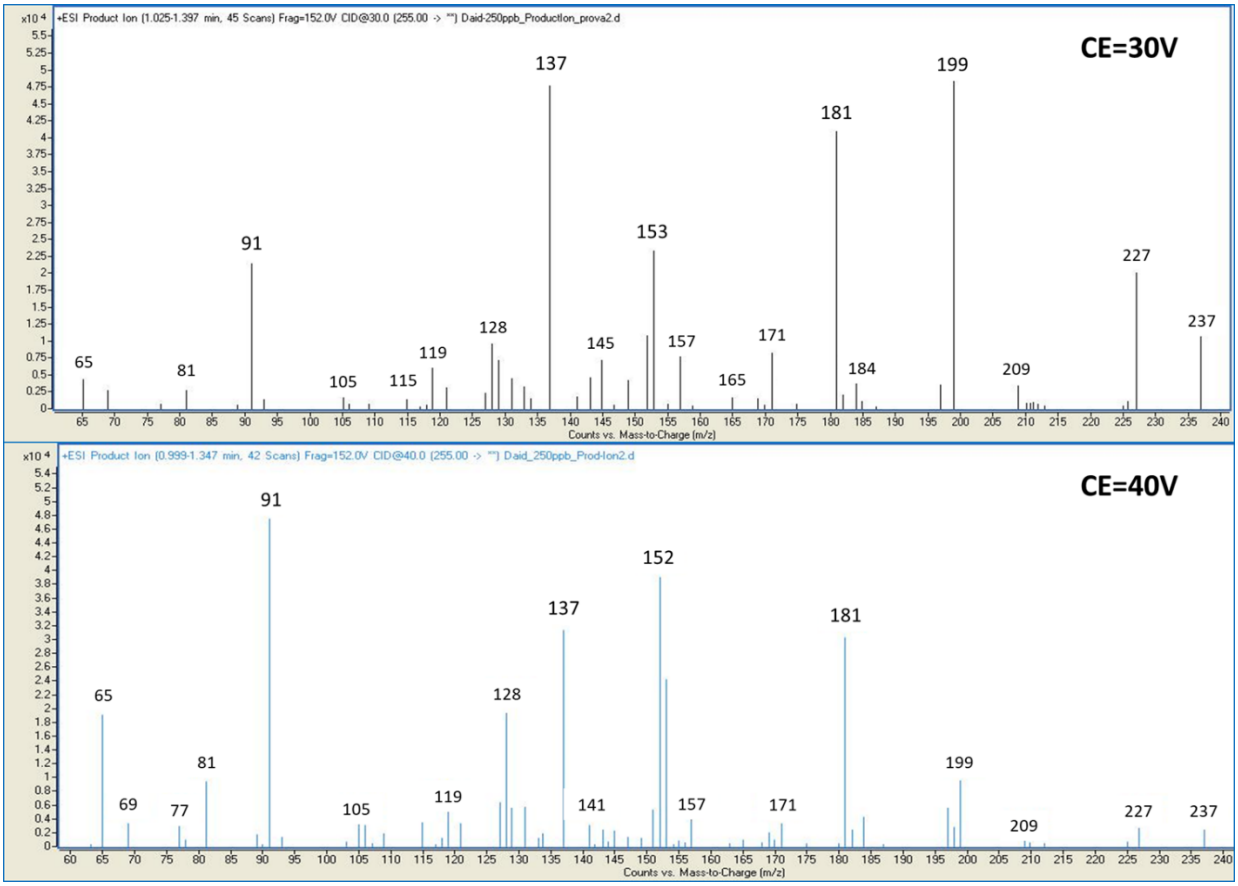


Figure 22: product ion spectra of daidzein, acquired at collision energies of 30 V and 40 V. The spectra derived from the fragmentation of the precursor ion at m/z= 255.

The most abundant product ions identified by the Optimizer were confirmed in the product ion spectra, and therefore used for quantitation and confirmation purposes. Table 9 reports the MRM transitions selected for each compound and the associated optimal values of the MS acquisition parameters.

Table 16: MRM transitions for the five phytoestrogens and optimized acquisition parameters; quantitative transitions are indicated in bold.

Compound	Precursor ion $[M+H]^+$ (m/z)	Product ions (m/z)	Dwell Time (ms)	Fragmentor voltage (V)	Collision Energy (V)	Cell Accelerator voltage (V)
DAID	255	**199**, 137, 91	100	152	**24**, 28, 40	7
GEN	271	153, **91**	100	152	28, **44**	7
COUM	269	**213**, 157	100	152	**28**, 36	7
FORM	269	253, **197**, 118	100	152	28, **44,** 32	7
BIOCH	285	**213**, 152	100	152	**40**, 24	7

Comparison of the GC-MS and LC-MS methods: figures of merit

The most important advantage of using LC-MS instead of GC-MS, except for the skipping of the derivation step, is the possibility to analyze all the five phytoestrogens. In fact, the main problem of the GC-MS method was the impossibility of validating and applying it to the reliable determination of biochanin A and genistein in the soy milk samples. Due to problems of stability of the derivatives, the chromatographic peaks' areas readily decrease after the first injection, hindering the performance of instrumental replicates and leading to rather poor repeatability. The application of the LC-MS method allowed to overcome this problem, and the validation was successful for all the five analytes. Another benefit was the reduction of the analysis time; the GC run lasted 30 minutes, while the HPLC run was around 30% more rapid, with a total of 19 minutes, including the post time. This gain becomes considerable when several samples must be analyzed.

The main figures of merit of the developed LC-MS/MS method were evaluated: linearity, detection and quantitation limits, precision and specificity. These values were compared with those obtained by the GC-MS/MS technique and all the parameters are reported in Tables 17 and 18.

Table 17: comparison of the LC-MS/MS and GC-MS/MS methods: linearity parameters of the calibration curves.

	R^2		Linearity range ($\mu g\ L^{-1}$)	
	GC-MS/MS	LC-MS/MS	GC-MS/MS	LC-MS/MS
FORM	0.9984	0.9952	8-500	0.5-200
DAID	0.9978	0.9976	8-500	0.5-200
COUM	0.9996	0.9986	8-500	0.5-200
BIOCH	0.9650	0.9998	50-250	0.5-200
GEN	0.9750	0.9993	50-250	0.5-200

Table 18: comparison of the LC-MS/MS and GC-MS/MS methods: limits of detection and quantitation, precision.

	LOD ($\mu g\ L^{-1}$)		LOQ ($\mu g\ L^{-1}$)		Intra-day Precision (RSD%)		Inter-day Precision (RSD%)	
	GC-MS/MS	LC-MS/MS	GC-MS/MS	LC-MS/MS	GC-MS/MS	LC-MS/MS	GC-MS/MS	LC-MS/MS
FORM	1.1	0.03	3.6	0.10	4.7	2.4	12.4	5.2
DAID	1.8	0.17	5.9	0.57	6.1	1.9	13.5	7.1
COUM	0.1	0.13	0.3	0.43	4.8	2.7	12.1	5.9
BIOCH	17.7	0.09	59.2	0.31	54.9	3.8	-	5.5
GEN	3.9	0.34	13.1	1.15	50.6	3.3	-	6.3

As far as linearity of the calibration curves is concerned (Table 17), the LC-MS method was characterized by linearity ranges shifted to lower concentration levels and involving a larger range, of almost 3 orders of magnitude; in fact, all phytoestrogens exhibited linear correlation among 0.5 and 200 $\mu g\ L^{-1}$. The GC-MS method showed linearity at higher concentrations and smaller range, of about two orders of magnitude, for FORM, DAID and COUM. If we look at the values for BIOCH and GEN, linearity was poor and not comparable to that obtained with the LC-MS method. The determination coefficients, used to evaluate the goodness of the calibration curve fitting, were comparable in the case of FORM, DAID and COUM, while definitely better for BIOCH and GEN when using LC-MS/MS.

As expected, the limits of detection and quantitation of the LC-MS/MS method were lower for the analyzed compounds, with the exception of COUM (Table 18). This compound exhibited excellent sensitivity with the optimized GC-MS/MS method, showing a slightly better LOD with respect to LC-MS/MS. For the other compounds, the increase in sensitivity obtained with the LC-MS/MS method was of one order of magnitude for GEN, DAID and FORM, and more than two orders of magnitude for BIOCH.

A noteworthy improvement was observed in repeatability and intermediate precision passing from the GC-MS to the LC-MS method. The RSD% of the intra-day replicate analyses by LC-MS (n=3) were half the values obtained with the GC method for FORM and COUM, while three times smaller for DAID. BIOCH and GEN could be analyzed with excellent precision by the LC-MS/MS method (RSD% below 4% for both compounds), in contrast with the extremely poor repeatability of the GC method. The inter-day assay showed better results as well, with RSD% of the LC-MS method equal or smaller than 7% for all compounds; inter-day RSD% of the GC-MS method were almost twice the values of the LC-MS method for FORM, DAID and COUM and not evaluable for BIOCH and GEN. The ratio among the validation parameters of the two techniques are summarized in Table 19, showing the overall improvement obtained by applying the LC-MS/MS method.

Table 19: Ratios of the figures of merit obtained with the LC-MS/MS and GC-MS/MS methods, calculated by putting the values for the LC method at the numerator.

Compound	LC/GC LOD ratio	LC/GC LOQ ratio	LC/GC Intra-day RSD ratio	LC/GC Inter-day RSD ratio
FORM	36.7	36.0	2.0	2.4
DAID	10.6	10.4	3.2	1.9
COUM	0.8	0.7	1.8	2.1
BIOCH	196.7	191.0	14.4	-
GEN	11.5	11.4	15.3	-

A final remark is on the specificity of the two methods. Thanks to the performance of tandem MS, both methods were characterized by high specificity; the reliable identification of the analytes was based on the combination of two criteria: the retention time (RT) and the presence of the chromatographic peak at the expected RT in the appropriate extracted ion current (EIC) chromatogram; the EIC is either referred to the current of selected precursor and product ions in the GC-ITQ method or the current of the MRM transitions in the LC-triple quad method. In addition, the ratio among the EIC peak area of two product ions (in the case of the GC-ion trap MS) or the ratio among the EIC of two MRM transitions (in the case of the LC-triple quad MS) were evaluated for confirmation: if the less intense signal was down to 10% of the most intense one, a maximum difference of 20% in the real sample with respect to the pure standards is acceptable for GC-MS/MS, while a maximum difference of 30% is acceptable for LC-MS/MS [105,106]. The acceptance criteria

were always verified in the analyzed samples. Both techniques are hence very specific and suitable for a reliable identification.

Comparison of the results of soy milk analysis

The same soy milk samples analyzed by the developed GC-MS/MS approach were subjected to LC-MS/MS analysis to verify the quantitative data obtained. The same methanol extracts obtained from the SPE procedure and analyzed by GC-MS were stored at -20°C and re-analyzed by LC-MS after two months. The results obtained for the three soy milk samples were not completely in accordance between the two methods, but this could be ascribed to possible degradation, depending on the different matrices. In fact, a non-homogeneous behaviour was observed going through the different samples. The comparison among the obtained quantitative data are reported in Table 20.

Table 20: comparison of the analysis results for soy milk samples.

	Soy milk 1 (mg L^{-1})		**Soy milk 2 (mg L^{-1})**		**Soy milk 3 (mg L^{-1})**	
	GC-MS/MS	LC-MS/MS	GC-MS/MS	LC-MS/MS	GC-MS/MS	LC-MS/MS
FORM	<LOD	0.00026 ± 0.00001	0.033 ± 0.003	0.0411 ± 0.0009	0.024 ± 0.007	0.00200 ± 0.00001
DAID	4.1 ± 0.9	3.31 ± 0.04	7 ± 1	5.25 ± 0.05	10.4 ± 0.7	2.07 ± 0.02
COUM	<LOD	<LOD	0.033 ± 0.002	0.00144 ± 0.00003	0.028 ± 0.004	0.00090 ± 0.00006
BIOCH	0.07 ± 0.04	<LOD	0.2 ± 0.1	0.00127 ± 0.00004	0.4± 0.2	0.00068 ± 0.00006
GEN	16 ± 8	4.8 ± 0.4	10 ± 6	9.0 ± 0.8	16 ± 8	2.0 ± 0.3

The uncertain results obtained for BIOCH and GEN make it difficult to compare the data for these two analytes. Nevertheless, a much lower concentration of both compounds was detected by the LC-MS/MS method in all the three samples; the only exception was GEN in sample 2, characterized by the same results. The general smaller concentrations found by LC-MS could be due to degradation; however, an over-estimation during the GC-MS analysis could not be excluded, because of carry-over problems observed for these two analytes.

As far as the other analytes are concerned, each sample will be discussed separately, since the difference in matrix composition could have caused differences in the sample conservation. In sample 1, results for FORM and COUM were in accordance among the two methods. In fact, FORM was detected by the LC-MS/MS method at the ng L^{-1} level, namely under the LOD of the GC method (1.1 µg L^{-1}), while COUM was under the LOD for both analyses. A slightly lower level of DAID was found by the LC-MS/MS method, even though still comparable with the GC-MS result. In sample 2, comparable concentrations were found for FORM and DAID, while a decrease in COUM level was

observed by the LC-MS/MS method. As for sample 3, the concentration of all the five compounds detected by the LC-MS/MS method, were rather low: up to three order of magnitude difference was observed, with FORM detected at 2 μg L^{-1} and COUM and BIOCH at the ng L^{-1} level. The great difference observed was in contrast with the other results, suggesting a probable massive degradation in sample 3.

Even though the results were partially not consistent between the two methods, the analysis of the same samples stored for a quite long period of time could have caused the main differences observed in the results. On the other hand, the possibility of detecting very low concentration of the considered analytes in soy milk by LC-MS was demonstrated, and the higher repeatability of this technique produced more reliable results, in particular for the determination of BIOCH and GEN.

The analysis of soy milk extracts highlighted an advantage of the GC-MS technique, namely the less probable occurrence of matrix effect. In fact, generally, the total ion current (TIC) chromatograms obtained by GC-MS analysis of soy milk samples were cleaner than the ones obtained by LC-MS analysis. Fig. 23 shows the TIC chromatogram of a soy milk sample (200-fold diluted aliquot) analysed by GC-MS and by LC-MS; the presence of several peaks in the LC-MS TIC chromatogram indicates the presence of many species other than the analytes, detected despite the use of the MRM mode. This may suggest that matrix effect is more likely to occur when analysing the soy milk extracts by LC-MS.

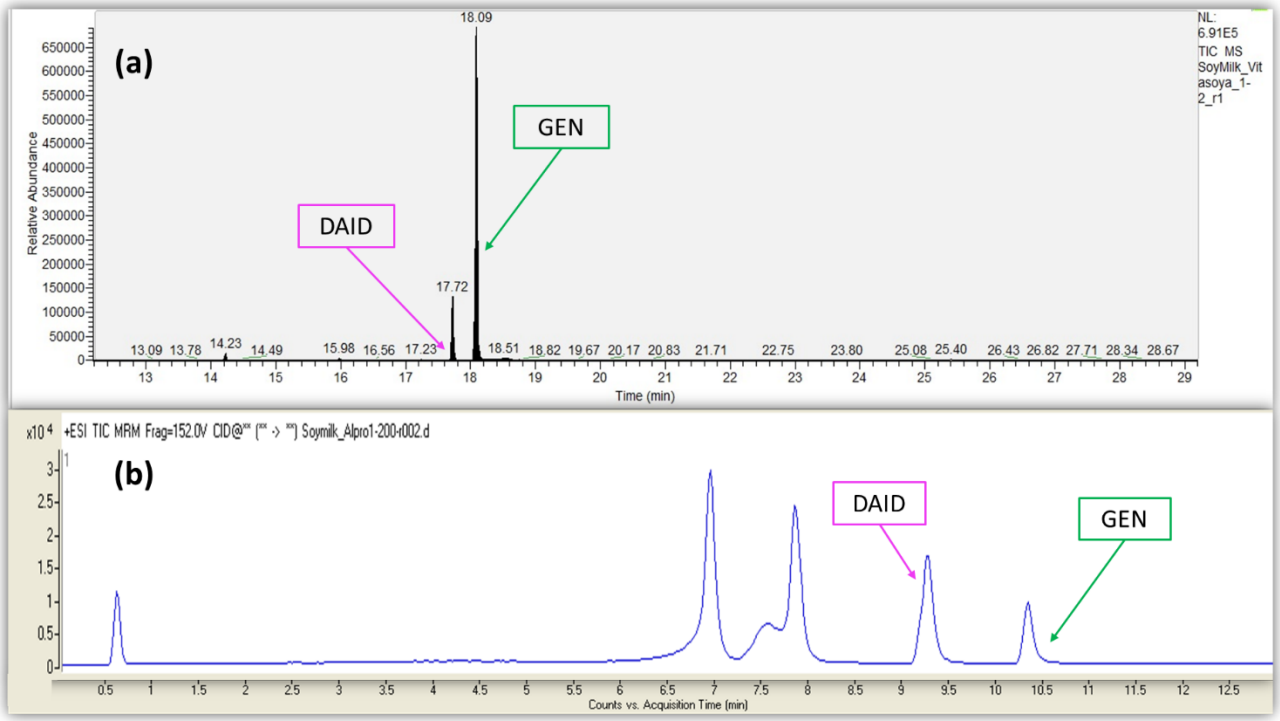


Figure 23: Total Ion current (TIC) chromatograms obtained after analysis of a soy milk sample (200-fold diluted aliquot) by GC-MS/MS (a) and HPLC-MS/MS (b).

4.4 Conclusions

The developed LC-MS/MS method was characterized by good figures of merit for all the considered analytes and proved to be suitable for the analysis of phytoestrogens in soy milk. The comparison with the GC-MS/MS method highlighted some major advantages of LC-MS over the other technique:

- derivatization is not necessary, leading to faster, easier and more reproducible analyses;
- the method can be considered validated for all the five compounds, overcoming the problems encountered in quantitation of GEN and BIOCH by GC-MS;
- the limits of detection were generally improved (except for COUM), with values of 0.03-0.34 µg L^{-1}, versus the higher limits reached with GC-MS (0.1-17.7 µg L^{-1});
- higher precision, both considering intra-day and inter-days assays, was reached;
- linearity ranges were larger and shifted to lower concentration levels, with excellent determination coefficients for all analytes;
- shorter analysis time.

On the other hand, both methods are characterized by high specificity; moreover, the derivatization technique, coupled with the GC analysis might lead to a less probable presence of interferent species, and, hence, minor matrix effect.

Overall, the HPLC-MS/MS method is preferable for the main advantages discussed and must be applied if reliable quantitation of GEN and BIOCH is needed. However, the GC-MS/MS method can be considered a valid alternative for quantitation of DAID, FORM and COUM, when the required LODs are not excessively low and more expensive instrumentation is not available.

Chapter 5

Comparative study of different extraction techniques for phytoestrogens analysis in soy-burgers by LC-MS

5.1 Introduction

In the current market several vegetal soy-based foods are spreading, proposed as healthy or vegan options. Alongside with the more classical soy derivatives, such as soymilk and tofu, more complex products are appearing; they are often burgers, cutlets or sausages based on soy preparations, with the addition of other vegetables and cereals. In the latest years, the consumption of these foods has increased, due to the diffusion of particular diets, which need substitutes of meat and dairy products. The long-term effects of consuming processed soy-based products have not been investigated so far, especially due to the relatively new appearance of such products in the international market. Therefore, it is important to determine the phytoestrogens content of these products, to estimate the resulting daily intake of specific classes of consumers.

Few data are present in the literature about the phytoestrogen content of soy-based meat substitutes [43,53] and usually these samples are included in a wide group of "soy foods". The analysis is usually performed by HPLC, coupled to mass spectrometry or diode array detectors. Various extracting methods are used for soy-based food. Ultrasound assisted extraction (UAE), pressurized liquid

extraction (PLE), microwave assisted extraction (MAE) and solid phase extraction (SPE) are the most used techniques for soy-based products and are usually applied to the determination of phytoestrogens in soy beans or simple soy derivatives. Some innovative techniques have been recently proposed, such as the Quick Easy Cheap Effective Rugged and Safe (QuEChERS) methodology [50]. When the same method is applied to different kind of samples, the results could be affected by errors related to different matrix effects; in particular, when using ESI mass spectrometry, matrix effect can appear as ion suppression or ion enhancement, due to other species present in the samples. Therefore, it is important to carefully estimate the matrix effect of each type of sample with specific tests which compare the response of the analytes in the matrix and in neat standards.

In the present chapter an improved LC-MS/MS method is presented for the determination of the five studied phytoestrogens, focusing on the analysis of soy-based meat substitutes. With respect to the LC-MS/MS method described in the previous chapter, a different type of chromatographic column was tested to improve the chromatographic conditions, including analysis time. Three techniques for the extraction of phytoestrogens from soy-based burgers prior to the LC-ESI-MS/MS analysis were tested and compared, in terms of recovery and matrix effect: the traditional UAE, UAE followed by SPE and a QuEChERS method. This study put the basis for a systematic optimization of the extraction procedure, in order to obtain maximum recovery and negligible matrix effect, permitting reliable quantitation of phytoestrogens in soy-based burgers and similar products.

The results presented in this chapter are object of a paper published in an international ISI journal (see Appendix: Publication 3).

5.2 Materials and methods

Chemicals

The phytoestrogens standards (DAID, GEN, FORM, BIOCH and COUM) were the same used in the previous parts of the work, purchased from Sigma Aldrich (St. Louis, MO, USA). Magnesium sulfate was purchased from Carlo Erba Reagenti (Rodano, MI, Italy) and sodium chloride was from Sigma Aldrich (St. Louis, MO, USA). End-capped C18 bonded silica loose sorbent was obtained from Supelco (Bellefonte, PA, USA).

Methanol (MeOH) and acetonitrile (ACN) were from VWR Chemicals (Fontenay-sous-Bois, France), while formic acid (98%) was obtained from Sigma Aldrich (Steinheim, Germany). All solvents were of chromatographic grade.

Ultra pure water was obtained from a Millipore Q-Gard system equipped with a Millipak 0.22 µm

filter (Millipore, Watford, Hertfordshire, UK).

Instrumentation and LC-MS analysis

The analyses were performed using the instrument already mentioned before: Agilent 1200 SL high performance liquid chromatograph coupled to an Agilent 6430 triple quadrupole mass spectrometer with an ESI ion source. The chromatographic column used in the proposed improved method was a pentafluorophenyl (PFP) column with core-shell particles (Kinetex PFP, 2.6 μm, 100 mm× 2.1 mm ID, by Phenomenex, Torrance, CA, USA). The best chromatographic separation was achieved within 9 min. The injection was of 10 μL, the column was kept at 40 °C, the flow was of 0.5 mL min^{-1} and the mobile phases were H_2O with 0.01% (v/v) of formic acid (phase A) and ACN (phase B). The elution gradient was characterized by an initial 90% of phase A, hold for 1 minute, an increase of phase B to 80% at minute 6, hold for 1 minute, and a return to the initial conditions in 1 minute, hold for 1 minute. An additional post run time of 4 min was added to completely re-equilibrate the column. The mass spectrometric conditions used were the same optimized and discussed in chapter 4 (MRM parameters reported in Table 16). The most abundant MRM transition was considered for quantitative analysis, while the other was used for confirmatory purposes, with the only exception of BIOCH. In the soy burger extracts a clear interferent with the same mass transition of BIOCH (m/z 285→213) was observed at an overlapped retention time. For this reason, the less abundant transition was used as the quantitative one for this analyte. Table 21 summarized the retention times of the improved HPLC-MS/MS method and the transitions used for the quantitation during this part of the thesis work.

Table 21: retention times and quantitative MRM transitions of the five analytes in the optimized method.

Compound	**Retention time (min)**	**MRM transition:** Precursor ion (m/z)→ product ion (m/z)
DAID	5.30	255 → 199
GEN	5.84	271 → 91
COUM	5.88	269 → 213
FORM	6.06	269 → 197
BIOCH	6.62	285 → 152

Extraction procedures

The comparison involved three different types of extraction with the following procedures:

- **Ultrasound Assisted Extraction**: a portion of 0.2 g of freeze-dried soy burger was weighted in a 15 mL plastic centrifuge tube, 5 mL of MeOH:H_2O (80:20, v/v) was added and the tube was put in an ultrasonic bath (model USC600D by VWR, Leuven, Belgium) at 30 °C for 15 min. The tube was centrifuged at 5000 rpm for 5 min (centrifuge model 4206 by ALC international, Milan, Italy) and the supernatant collected. The solid residue was extracted a second time with the same procedure using 2.5 mL of solvents mixture. The supernatant fractions were combined and frozen overnight at -18 °C; this step could provide a clean-up from high molecular weight species, which precipitate at low temperature [40]. The extract was then filtered through a 0.2 µm polytetrafluoroethylene (PTFE) filter (Whatman, Maidstone, UK) and analyzed by HPLC-MS/MS twice: directly and 100-fold diluted with MeOH.
- **Solid Phase Extraction**: the extract obtained with the UAE technique was subjected to Solid Phase Extraction (SPE), by applying the method developed in our laboratory for soy milk [107], with some modifications. 1.5 mL of the UAE extract was dried under N_2 and reconstituted with 15 mL of H_2O. A 200 mg Oasis HLB (Hydrophilic Lipophilic Balanced) cartridge (Waters, Dublin, Ireland) was conditioned with 4 mL of MeOH and 4 mL of H_2O and the 15-mL sample was loaded. Then, the cartridge was washed with 6 mL of H_2O and dried under vacuum. The elution was performed with 3 mL of MeOH and the eluate dried under a N_2 stream. The residue was re-dissolved in 1.5 mL of MeOH and filtered through a 0.2 µm PTFE filter; the obtained solution was analyzed by HPLC-MS/MS twice: directly and 100-fold diluted with MeOH.
- **QuEChERS methodology**: the QuEChERS technique was developed starting from the protocol employed by Bustamante-Rangel et al [50] on soy-based biscuits. 0.2 g of freeze-dried soy burger was weighted in a 15 mL plastic centrifuge tube, 10 mL of ACN:H_2O (50:50, v/v) was added and manually shaken for 5 min. Then, 4 g of $MgSO_4$ and 1 g of NaCl were added, vigorously shaken for 1 min and centrifuged at 5000 rpm for 5 min; this step allows the phase separation of ACN and water, thanks to the salting out effect. 1 mL of organic layer was subjected to a clean-up step: dispersive SPE was performed with 150 mg of $MgSO_4$ and 25 mg of C18 loose sorbent, again shaking vigorously for 1 min. The tube was centrifuged at 5000 rpm for 5 min, the supernatant collected and an aliquot of 800 µL was dried under N_2. After reconstituting with the same volume of MeOH, the extract was filtered through a 0.2

µm PTFE filter and the obtained solution was analyzed by HPLC-MS/MS twice: directly and 100-fold diluted with MeOH.

Calibration and figures of merit

Calibration was performed preparing standard solutions in MeOH at six concentration levels and analyzing them in triplicate: 0.5 µg L^{-1}, 2 µg L^{-1}, 10 µg L^{-1}, 50 µg L^{-1}, 100 µg L^{-1} and 200 µg L^{-1}. Intra-day (6 replicates) and inter-day precision (4 days, 3 replicates per day) were evaluated for each level of the calibration curve; limit of detection (LOD) and limit of quantitation (LOQ) of the analytes were calculated considering a signal to noise ratio of 3 and 10 respectively. The noise signal was considered as the average integration value of background regions with the same width of the analytes' peaks. The evaluation of the average noise was performed for each analyte's MRM quantitative transition. Once LODs were estimated, a standard solution containing each compound at its LOD concentration was analyzed for confirmation; the chromatogram obtained after analyzing this solution and extracting the MRM signals, is shown in Fig. 24.

Figure 24: Extracted MRM transitions of the five analytes at the LOD concentration.

The accuracy of the whole analytical method, (sample pre-treatment plus instrumental analysis) was assessed, determining recovery of the three proposed extraction procedures; precision was evaluated as well, performing six replicates for each extraction technique. No reference materials are available

for phytoestrogens in soy-based burgers, therefore recovery was evaluated according to Matuszewski et al [87]. The spike concentration levels used to evaluate recovery were near to those expected in the samples, namely 50 µg g^{-1} for GEN, 25 µg g^{-1} for DAID, 0.075 µg g^{-1} for FORM and COUM and 1.25 µg g^{-1} for BIOCH. Each extraction procedure was performed in triplicate both on spiked and non-spiked soy burger; samples spiked before extraction were compared to samples spiked after extraction and average recoveries were determined. The quantitation of phytoestrogens in real samples was performed with the external calibration method, using the chromatographic area associated to the quantitative MRM transitions.

Burger samples

Soy-based burgers were purchased in an Italian drugstore. Four samples with different composition and soy amount were analyzed, comparing UAE and QuEChERS pre-treatments. Before extraction, samples were minced and freeze-dried by a lyophilizer (model DW1-060E by Heto-Holten, Allerød, Denmark). The freeze-dried material was further ground with a mortar to obtain homogeneous samples and kept at -18 °C until extraction. After sample treatment, the extracts were analyzed by HPLC-MS/MS.

5.3 Results and Discussion

Improvement of the HPLC-MS/MS method

The HPLC method developed for the analysis of the five phytoestrogens in soy milk surely improved the previous GC method, in terms of total analysis time, sensitivity and repeatability. Nevertheless, the quite complicated gradient used to obtain separated peaks of acceptable shape led to a chromatographic run of 19 minutes, including the post time. We decided to try to shorten the analysis time, and since all the chromatographic factors were deeply studied for the C18 column, the test of another stationary phase seemed a useful option.

The pentafluorophenyl (PFP) bonded silica stationary phase was considered a valid alternative to the C18 phase, thanks to its characteristics, theoretically suitable for a better retention of slightly polar compounds, aromatic and unsaturated compounds and phenols. Fig. 25 shows the structure of a classical C18 phase, compared with the PFP one.

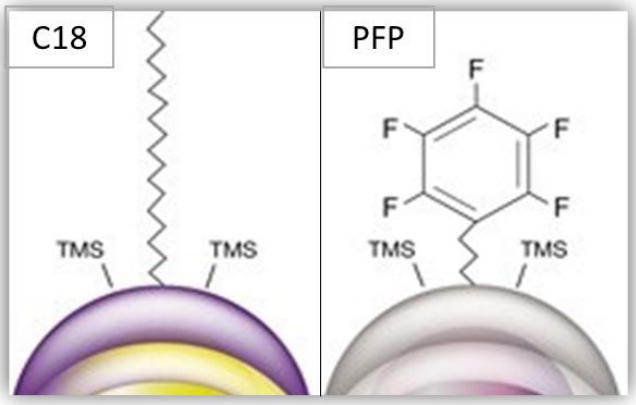


Figure 25: C18 and PFP stationary phases: comparison of the chemical structure of the binding sites.

PFP-bonded phases provide different mechanisms of interaction, including hydrophobic, π-π interaction, dipole-dipole, H-bonding and shape selectivity. For this reason, they are considered versatile phases, of medium polarity, and particularly suitable for binding aromatic moieties by the π-π interaction. The phytoestrogens studied are basically polyphenolic compounds of medium polarity, therefore the characteristics of the PFP phase seemed particularly appropriate for their efficient separation. The column tested was a Kinetex PFP, of 100 mm length, 2.1 mm internal diameter (ID) and packed with core-shell particles of 2.6 μm, by Phenomenex. Besides the different chemical composition of the phase, another substantial different of this column with respect to the C18 Zorbax one, was the packing with the core-shell particles. These particles are made of a solid core, covered by a porous outer shell and can reduce diffusion of the analytes, providing better mass transfer and resolution, at lower backpressure [108].

Thanks to the core-shell property and the consequent lower pressure given by the column, the mobile phase flow, as well as column temperature, were the parameters immediately taken into account to shorten the rum time. Moreover, a new study of the chromatographic gradient was necessary, due to the different interaction mechanisms among the analytes and the stationary phase, which could possibly lead to better retention properties. All trials were performed analyzing a standard solution of the five analytes at a concentration of 50 μg L^{-1} in MeOH. Mobile phase flow and column temperature were explored in the range 0.4-0.6 mL min^{-1} and 25-50 °C, respectively. The first gradient tested was the one optimized for the C18 column: the results were very satisfying, with a significant improvement of peak shape and excellent separation achieved. Therefore, simpler and more rapid gradients were evaluated, in order to reach separation in a shorter run, with no loss in separation efficiency.

The best conditions were achieved at 40 °C with a flow of 0.5 mL min^{-1} and using the elution gradient reported in the "Instrumentation and experimental conditions" section. Fig. 26 highlights the clear

improvement obtained with the use of the PFP stationary phase: sharper and more symmetric peaks were obtained for all compounds if compared to the ones obtained with the C18 column.

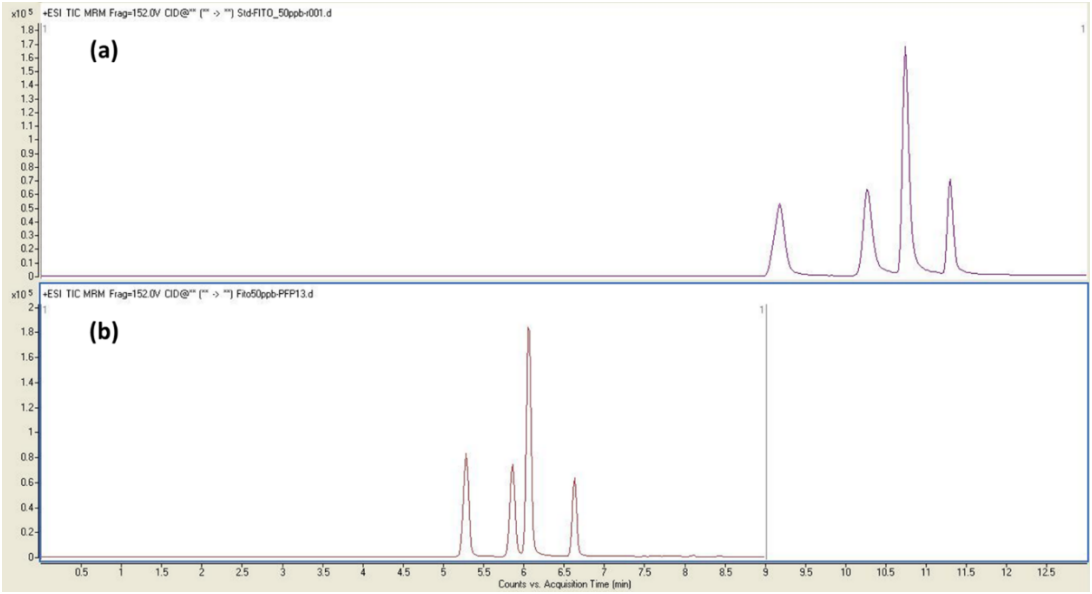


Figure 26: comparison of the chromatographic separation obtained with the C18 (a) and the PFP (b) stationary phases, applying the different gradients optimized for each column.

Although the efforts made to optimize the chromatographic run, the coelution of GEN and COUM was observed once again. Nevertheless, the use of the MRM mode made this separation unnecessary. The same MRM transitions and parameters already described in the previous chapter were used in mass spectrometric detection. Fig. 27 shows the overlapped extracted currents of all MRM quantitative transitions, indicating the possibility of resolving the 5 substances within 9 minutes.

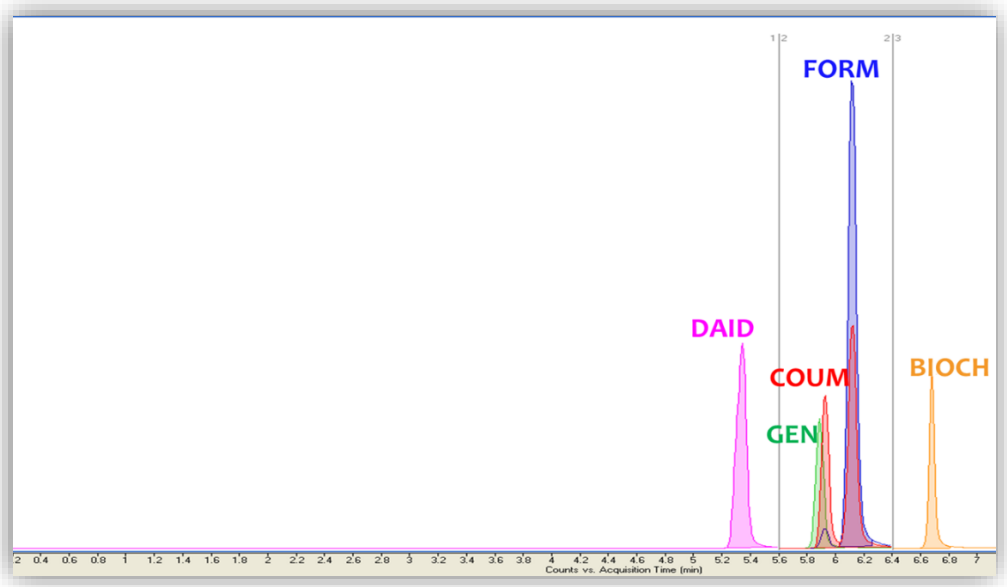


Figure 27: extracted currents of the MRM transitions for the five phytoestrogens. Chromatographic separation performed by the use of the PFP column and optimal gradient.

Analytical performances

The main figures of merit of the new instrumental method were evaluated: linearity range, LODs and LOQs, inter-day and intra-day precision. The values of these parameters for the five phytoestrogens are summarized in Table 22.

Table 22: figures of merit of the new instrumental method for the five analytes.

Compound	R^2	Linearity (µg L^{-1})	LOD (µg L^{-1})	LOQ (µg L^{-1})	Intra-day Precision (RSD%)	Inter-day Precision (RSD%)
DAID	0.9943	0.5-200	0.09	0.3	3.0	12.7
GEN	0.9997	0.9-200	0.3	0.9	3.5	9.3
COUM	0.9985	0.5-200	0.06	0.2	2.5	8.6
FORM	0.9951	0.5-200	0.02	0.07	2.1	8.6
BIOCH	0.9998	0.5-200	0.09	0.3	3.3	5.7

The linearity was verified in the range of 0.5-200 µg L^{-1} for all compounds, except for GEN (0.9-200 µg L^{-1}), achieving good determination coefficient (R^2) for all calibration curves (>0.99). The MRM method provided a very good sensitivity, with LODs and LOQs in the range of 0.02-0.3 µg L^{-1} and 0.07-0.9 µg L^{-1} respectively.

Intra-day and inter-day precision were assessed at all concentration levels of the calibration curve; the average values obtained, expressed as relative standard deviation (RSD), were in the range 2.1-3.5% and 5.7-12.7% respectively. With respect to the other HPLC method, all validation parameters were comparable, and even better sensitivity was reached for COUM, BIOCH and DAID. The only drawback in using this new method was inter-day repeatability, for which the new method gave slightly worse results. However, all values were acceptable and generally below a RSD of 10%.

Comparison of the extraction procedures

The extraction of phytoestrogens from soy-based burgers was performed with three extraction methods: ultrasound assisted extraction (UAE), UAE followed by solid phase extraction (SPE) and QuEChERS technique. Soy burgers are constituted of a mixture of soy or soy derivatives (tofu, texturized soy, soy juice), vegetables and cereals; because of the complexity of the matrix, interferences may affect the determination of phytoestrogens, despite the application of the specific HPLC-MS/MS method with MRM mode. Therefore, we wanted to compare a simple sample treatment which did not include any purification step (UAE) with two procedures which consisted of solvent extraction followed by a clean-up step (SPE and QuEChERS).

In the first technique, UAE, the variables involved, such as solvent, time and temperature, were chosen on the basis of techniques used to extract isoflavones from other soy-based matrices [35,39,40,109,110]. Two consecutive extractions were performed to have a compromise between quantitative extraction and rapidity of the analysis. The second technique involved the same UAE, followed by SPE purification on an Oasis HLB cartridge, which is widely used for the elimination of the most common interferents, such as sugars and lipids. The protocol used was similar to the one developed in our laboratory for soy milk samples, which gave acceptable recovery values for the analytes under consideration.

As for the QuEChERS method, two optimized approaches for the determination of isoflavones in legumes and soy-biscuits [48,50] were considered as starting point to develop the protocol. The two methods differ in the proportion of ACN and water used in the solvent mixture and for the clean-up step, that was not applied to legumes samples. In the first step of the original QuEChERS method [61], pure ACN is used as extraction solvent. The protocol was intended for the treatment of fruit and vegetables, which naturally have high percentage of water: a separation of the aqueous and organic phases occurs due to the addition of $MgSO_4$ and NaCl. The "salting out" effect favors the dissolution of the analytes in the organic portion and allows a first purification from the most polar interferents, which pass in the aqueous fraction. Since the freeze-dried burger samples did not contain any water, a mix of ACN:H_2O in a ratio of 50:50 (v/v) was chosen; higher percentage of aqueous solvent should be avoided to ensure dissolution of the analytes in a sufficient volume of organic solvent. We decided to apply the complete QuEChERS methodology, therefore including a clean-up step; C18 sorbent was used in a dispersive SPE, in order to eliminate the most lipophilic interferents. Volume of solvent and amount of clean-up sorbent was the same used by Bustamante-Rangel et al [50], but we chose a smaller amount of sample, since we expected higher concentrations of phytoestrogens in our sample with respect to soy-biscuits, given the higher content of soy in soy-based burgers.

The accuracy and precision obtained with the three proposed techniques were established in terms of recovery, matrix effect and procedural precision.

Recovery

A soy burger sample with an intermediate soy content was used to evaluate recovery of the extraction procedures. Starting from literature data, we assumed that the amount of phytoestrogens was roughly related to the percentage of soy present in the matrix. On this basis, we estimated the concentration of the five analytes in soy burgers with a percentage of soy around 35-45%, which was in the order of tens of ppm for DAID and GEN, tens of ppb for FORM and COUM and hundreds of ppb for BIOCH. DAID and GEN expected concentrations would probably fall out of the linearity range of

the calibration curves; for this reason, the analysis of each extract was always performed twice: directly for the determination of COUM, FORM and BIOCH and after a 100-fold dilution for the determination of DAID and GEN. Recovery was assessed after HPLC-MS/MS analysis of a non-spiked sample (NS), a sample spiked before extraction (B) and a sample spiked after extraction (A). Percent recovery was calculated for each compound through the following formula:

$$R\ (\%) = \ 100 * \frac{PA_B - PA_{NS}}{PA_A - PA_{NS}}$$

where PA_B, PA_{NS} and PA_A are the peak areas obtained by analyzing extracts B, NS and A, respectively. This workflow was followed for the three different sample treatments and Fig. 28 summarizes the obtained results.

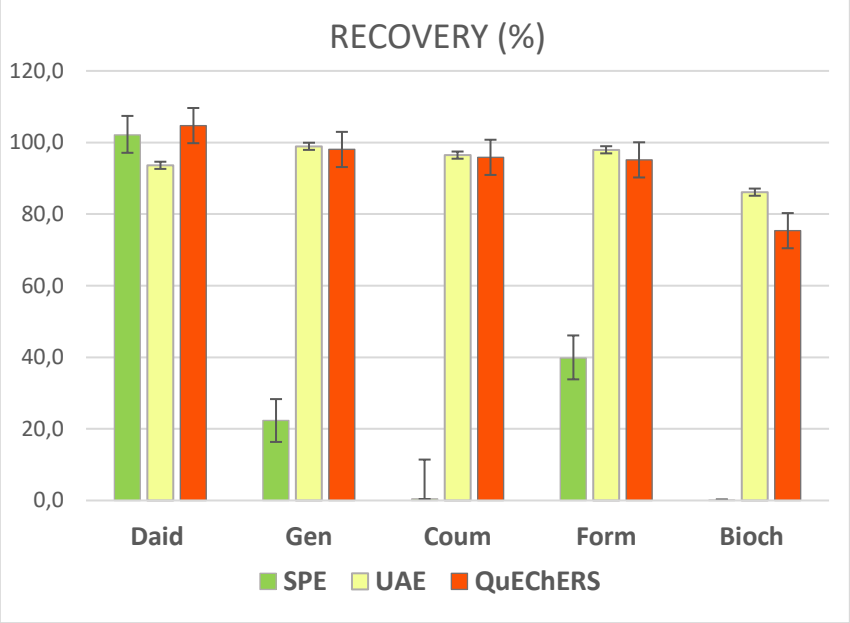


Figure 28: comparison of the recoveries (%) obtained with the three extraction procedures.

There was a substantial difference between the SPE procedure and the other two methods. In fact, except for DAID, which had a high recovery value in all cases, after the SPE purification the other analytes were poorly recovered (R=47% for GEN and R=40% for FORM) or completely lost (COUM and BIOCH). UAE gave very good results for all analytes, with average recoveries of 94-99% for DAID, GEN, COUM and FORM and 86% for BIOCH. The QuEChERS method led to comparable results: recoveries of 95-105% were reached for DAID, GEN, COUM and FORM, while a slightly lower value was found for BIOCH (R=75%).

Matrix effect

Matrix effect is commonly observed when complex matrices are analyzed, and many species with a range of chemical properties are present in the same sample. Matrix effect can either manifest as ion

suppression and, less frequently, ion enhancement. Ion suppression in the ESI source occurs when coeluted interferent species, which are more easily ionized, compete with the analytes ionization process. A wide range of mechanisms, which depend on the nature of the interferent species as well as on the experimental conditions, a suppression of the signal given by the analyte of interest is observed, leading to an underestimation of the actual concentration in the sample [111]. As for ion enhancement, it can be caused by species which somehow favor the ionization of the analytes of interest. The mechanisms which underlie this process are still not completely understood.

Matrix effect (ME) was evaluated by comparing the signal given by each compound in the extracts spiked after extraction (A) and in pure standard solutions in MeOH (P). The following formula was employed:

$$ME\ (\%) = \ 100 * \frac{PA_A - PA_{NS}}{PA_P}$$

where PA_A, PA_{NS} and PA_P are the peak areas obtained by analyzing extracts A, NS and the standard solution P, respectively. The results are shown by the graph in Fig. 29 and clearly highlight that a non- negligible matrix effect was observed in some cases with a different behaviour of each analyte.

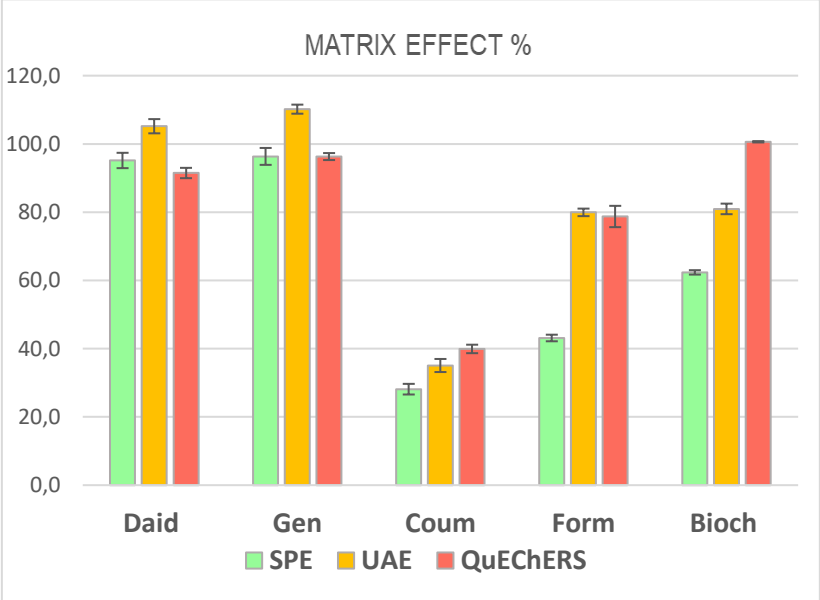


Figure 29: comparison of the matrix effect (%) observed in the extracts derived from the three pre-treatment methods. DAID and GEN were determined in the diluted extracts while COUM, FORM and BIOCH were determined in the non-diluted extracts.

For the given definition of the ME it is clear that ion suppression (%) is equal to 100-ME(%). Therefore, a value close to 100% indicate that no matrix effect is observed, a value smaller than 100% indicate ion suppression, while a value higher than 100% indicate ion enhancement. In the light of this, the higher ME% was observed for DAID and GEN which are the analytes determined by the analysis of diluted extracts; this clearly indicates the beneficial effect of dilution on the possible influence of the interferents. Only GEN exhibited a slight ion enhancement (ME=110%) when no

purification was performed (UAE). As for the other analytes, determined in the non-diluted extracts, a greater ion suppression was observed. Better results were obtained with the C18 clean-up or with no clean-up at all (simple UAE), with respect to the SPE purification. Even though it is supposed to limit the presence of matrix compounds, the SPE technique led to ME values of 28%, 43% and 62% for COUM, FORM and BIOCH, respectively; this indicates that problems in the ionization or signal stability are still present, despite passing the extracts through the Oasis HLB phase; it is possible that the SPE procedure enrich other species jointly with the analytes, causing problems of ion suppression. The results obtained by the UAE and QuEChERS methods showed a similar ME for COUM and FORM, of around 35% and 80% respectively. Finally, BIOCH resulted slightly suppressed in the UAE extracts (ME=80%), while no matrix effect was encountered when QuEChERS technique was used (ME=101%).

Precision

The precision of the extraction techniques was evaluated by performing six procedural replicates of the same sample for each method. The evaluation was on the entire procedure, thus including errors given by the experimental operations and instrumental variability. The values of RSD% are reported in Table 23.

Table 23: Precision of the three extraction methods expressed as relative standard deviation (RSD) with respect to 6 procedural replicates.

	RSD (%)				
Compound	**DAID**	**GEN**	**COUM**	**FORM**	**BIOCH**
SPE	3.3	17.7	32.5	6.0	50.8
UAE	2.0	2.6	6.4	2.8	2.6
QuEChERS	2.2	3.1	10.2	5.6	3.0

Best results were achieved when only UAE was performed, with RSD of 2-6% for all analytes; this was expected, since this procedure involves the minimum sample manipulation, limiting casual error. Nevertheless, also QuEChERS extraction gave good results, with RSD values of 2-10%. The lowest precision was found for COUM in both cases and it is noteworthy that this compound was the one most affected by matrix effect. The SPE procedure was characterized by extremely low precision for GEN, COUM and BIOCH, while repeatability was good for DAID and FORM.

To summarize, the results of the experiments demonstrated that both UAE and QuEChERS are suitable methods for the sample treatment of soy-based burgers and are characterized by high

recoveries of the five analytes and very good precision. As far as matrix effect is concerned, the QuEChERS method presented slightly better results for the analytes determined in the non-diluted extracts, with lower ion suppression for COUM compared to the other techniques and no ion suppression at all for BIOCH.

Application to soy burger samples

Considering the results obtained by the recovery tests, the UAE and QuEChERS were chosen to determine the phytoestrogens content of four soy burger samples. In order to perform the quantitation on real samples, the limit of detection and quantitation of the complete method (extraction plus instrumental analysis) were estimated, taking into account the average background noise in the chromatogram of the analyzed samples. The values, expressed with respect to the mass of the soy-burgers are reported in Table 24.

Table 24: LODs and LOQs expressed in µg g^{-1} of soy-burger sample.

Compound	LOD (µg g^{-1})	LOQ (µg g^{-1})
DAID	0.015	0.049
GEN	0.056	0.187
COUM	0.006	0.021
FORM	0.002	0.005
BIOCH	0.010	0.032

We decided to apply both procedures to the four samples, to verify if changes in the matrix composition would affect the quantitation of the analytes, using the external calibration method. Table 25 shows the main ingredients and the percentage of soy in the four commercial samples.

Table 25: percentage of soy and other ingredients of the soy burger samples analysed.

Sample	Soy content (% w/w)	Vegetables	Cereals	Other ingredients
1	23%	Zucchini, potatoes, leek	Wheat	Sunflower oil, salt, spices, additives
2	47%	Tomato, olives, carrots, potatoes, celery	Wheat	Sunflower oil, salt, spices, additives
3	19%	Carrots, onion, potatoes	Millet, corn	Sunflower oil, olive oil, salt, spices
4	33%	Onion, tomato	/	Sunflower oil, salt, sugar, vegetable fibers, additives

The same amount of freeze-dried material (0.2 g) was treated with the two extraction procedures and the results were normalized for the volume of solvent used. Results of the quantitation are shown in Table 26 and express the content of phytoestrogens in the dry mass, i.e. the lyophilized samples.

Table 26: Concentration levels of phytoestrogens determined in the freeze-dried soy burgers by using the UAE and QuEChERS methods.

	Sample 1 (µg g^{-1})		**Sample 2 (µg g^{-1})**		**Sample 3 (µg g^{-1})**		**Sample 4 (µg g^{-1})**	
	UAE	**QuEChERS**	**UAE**	**QuEChERS**	**UAE**	**QuEChERS**	**UAE**	**QuEChERS**
DAID	43 ± 3	41 ± 2	55 ± 3	59 ± 2	47 ± 3	49 ± 2	2.1 ± 0.1	2.4 ± 0.1
GEN	45.6 ± 0.4	38.1 ± 0.3	63.8 ± 0.4	71.9 ± 0.2	56.1 ± 0.4	58.3 ± 0.2	2.25 ± 0.03	2.55 ± 0.02
COUM	< LOD	< LOQ	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
FORM	0.006 ± 0.004	0.026 ± 0.003	0.008 ± 0.005	0.005 ± 0.003	< LOQ	< LOQ	< LOD	< LOD
BIOCH	< LOQ	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD

Despite the sensitivity of the method was in the low ng g^{-1} level for all analytes, COUM, FORM and BIOCH were almost always under the limit of detection or quantitation. Therefore, it was not possible to compare the efficiencies of the extraction methods for these analytes in all the four matrices. On the other hand, DAID and GEN were found at high concentrations, especially in sample 1, 2 and 3. No differences were found in the DAID quantitation using UAE and QuEChERS methodologies, indicating that the different matrix components did not influence DAID determination. As for GEN and FORM quantitation, sample 1 and 2 compositions seemed to influence the extraction recovery and matrix effect of UAE and QuEChERS. In fact, QuEChERS method was more effective in extracting FORM from sample 1 and GEN from sample 2.

It is noteworthy that no correlation was found between the soy percentage and the total phytoestrogens content of the four samples; this was already observed for the soy milk samples, discussed in chapter 3. We suppose that the variety of soy and the manufacturing process may affect phytoestrogens concentrations. Moreover, the other ingredients of the soy burgers may contribute to the total phytoestrogens content.

5.4 Conclusions

A faster and more sensitive HPLC-MS/MS method for the determination of the five phytoestrogens in the complex matrix of soy-based meat substitutes was developed. The method is characterized by a 9 min chromatographic separation and presents high specificity, repeatability and sensitivity. The comparison of three proposed extraction techniques, performed in terms of recovery, matrix effect and precision, showed that both simple UAE and the QuEChERS methodology are suitable for the quantitative analysis of the five analytes in soy burgers. On the contrary, SPE should be avoided in the treatment of this kind of samples, since it is characterized by low precision and recovery. All the three techniques showed a negligible matrix effect when diluted extracts were analyzed; on the other hand, ion suppression was always observed for COUM and FORM in the concentrated extracts. Further investigation is necessary to improve the clean-up method and avoid problems of ionic suppression. Moreover, considering the relatively high concentration of daidzein and genistein found in the considered samples, a broad study of the phytoestrogen content in soy burgers present on the market is desirable.

Chapter 6

Multivariate optimization of the extraction procedure for the analysis of phytoestrogens in soy-burgers

6.1 Introduction

As already highlighted in the previous chapter, accurate quantitation in complex food matrices is a challenging task. Alongside with the optimization of the instrumental analysis, a proper study of the pre-treatment is fundamental to obtain reliable quantitative results. The univariate approach (one variable at a time, OVAT) remains the most usual strategy for procedure optimization; nevertheless, if several variables are involved, its application is tedious and time-consuming, bringing only local knowledge of the system and ignoring interactions among variables. On the other hand, experimental design is a powerful tool which exploits the multivariate approach to obtain a reliable optimization. As far as the analysis of soy-food is concerned, two examples are present in the literature about the application of experimental design to optimize isoflavones extraction from soy-foods: the methods proposed were ionic liquid-based ultrasound-assisted extraction [53] and natural deep eutectic

solvents- extraction [52], while analysis was performed by HPLC-DAD. In these works, a non-soy matrix, taken as blank, was used for procedure optimization in terms of recovery, and the final method was applied to different soy products. When HPLC-ESI-MS is used for the analysis of complex matrices, both recovery and matrix effects should be considered in the optimization of the sample preparation; in fact, ion suppression or enhancement, due to matrix interferents, are highly probable and the study of the purification step during sample treatment becomes essential. No studies are present in the literature about the optimization of the extraction technique for the analysis of phytoestrogens in soy burgers. Most of the times this matrix is included in a list of general soy foodstuff and extraction methods developed for other matrices are applied to these samples [112]; moreover, the OVAT approach is commonly followed.

The modified QuEChERS procedure introduced in the previous chapter for the treatment of soy burgers demonstrated high potential to obtain a fast, easy and efficient extraction and purification of phytoestrogens. However, the necessity of a deeper study to improve the methodology for this specific matrix was highlighted.

In this framework, a comprehensive and thorough optimization of a QuEChERS method was accomplished for the determination of the five studied phytoestrogens in soy-based burgers by HPLC-MS/MS. The multivariate approach was exploited to optimize both recovery and matrix effect of the five compounds, by two sequential experimental designs.

6.2 Materials and methods

Chemicals

The phytoestrogens standards (DAID, GEN, FORM, BIOCH and COUM) were the same used in the previous part of the work, purchased from Sigma Aldrich (St. Louis, MO, USA). Magnesium sulfate was purchased from Carlo Erba Reagenti (Rodano, MI, Italy) and sodium chloride was from Sigma Aldrich (St. Louis, MO, USA). End-capped C18 bonded silica loose sorbent was obtained from Supelco (Bellefonte, PA, USA); Florisil and Primary Secondary Ammine (PSA) loose sorbents were purchased from Phenomenex, (Torrance, CA, USA). Methanol (MeOH) and acetonitrile (ACN) were from VWR Chemicals (Fontenay-sous-Bois, France), while formic acid was obtained from Sigma Aldrich (Steinheim, Germany). All solvents were of chromatographic grade. Ultra-pure water was obtained from a Millipore Q-Gard system equipped with a Millipak 0,22 µm filter (Millipore, Watford, Hertfordshire, UK).

Instrumental analysis

The method used for the determination of the five phytoestrogens was the one optimized and validated in the previous work. The analyses were performed using the instrument already mentioned before: Agilent 1200 SL high performance liquid chromatograph coupled to an Agilent 6430 triple quadrupole mass spectrometer with an ESI ion source. The chromatographic column used was the pentafluorophenyl (PFP) column and chromatographic separation was achieved within 9 min with the conditions described in chapter 5.

The mass spectrometric conditions used were the same optimized and discussed in chapter 4 (MRM parameters reported in Table 16). The MRM transitions used for quantitative purposes and the retention times of the analytes were reported in Table 21 (chapter 5).

Soy-burger sample pool

Three soy-based burgers with different ingredients and soy amount were purchased in an Italian drugstore; they were used to create a sample pool, in order to have an estimation of average matrix effect, which could slightly change due to the small differences in matrix composition.

The three samples were minced, freeze-dried and further ground altogether with a pestle to obtain a homogeneous pooled sample. The pool was constituted of an equal weight of the three components. The burger mix was stored at -18 °C during a maximum time of three months. All the multivariate optimization was performed by using the same pool, in order to avoid a source of variability which could affect the results of the experimental designs.

Experimental design

The multivariate approach of experimental design was adopted to optimize the extraction and purification steps of the sample preparation. The responses that we wanted to maximize were recovery and matrix effect of the sample treatment. The strategy followed for optimization was the performance of two consecutive experimental designs: a screening and a response surface design. In both designs, the response used to evaluate recovery was the peak area of the analytes, while matrix effect was estimated by the ratio among the signal given by the analyte in the matrix and in neat standard. For each experiment of the designs, two aliquots of the extract were analysed by LC-MS/MS: a non-diluted aliquot to determine the less concentrated analytes (COUM, FORM and BIOCH) and a 130-fold diluted aliquot to determine the most concentrated analytes (DAID and GEN). This double analysis was performed to resemble the procedure followed for quantitation of

real samples; in fact, the high concentration of DAID and GEN, makes it necessary to perform a dilution to avoid going out of the linearity range of the calibration curve.

The selected screening design was the Plackett-Burmann [68], used to investigate the effects of several variables on the selected responses for the five phytoestrogens. The extraction/purification procedure was based on the QuEChERS methodology; the extraction step involved the addition of an ACN:H_2O solvent mixture to the sample, agitation and addition of salts ($MgSO_4$ and NaCl) for the phase separation; the organic fraction was then collected and a dispersive solid phase extraction was used for clean-up ($MgSO_4$ and sorbents). Seven variables could potentially affect recovery and matrix effect of the analytes: volume of solvent mix/sample mass ratio (V_{solv}/m_{sample}), extraction time (shaking), PSA clean up sorbent, C18 clean up sorbent, Florisil clean up sorbent, clean up time (shaking) and shaking mode (ultrasound or manual). Two levels were selected for each variable (the lower coded as -1 and the upper coded as +1) and are presented in Table 27.

Table 27: Factors investigated in the Plackett-Burman screening design and selected levels.

Factor number	Factor	-1 level	+1 level
1	V_{solv}/m_{sample} (mL/mg)	0.025	0.05
2	Extraction time (min)	3	7
3	PSA sorbent (mg)*	0	50
4	C18 sorbent (mg)*	0	50
5	Florisil sorbent (mg)*	0	50
6	Clean-up time (min)	1	3
7	Shaking mode	manual	ultrasounds

*clean-up performed on 2 mL of extract

The Plackett-Burman design allows to explore the effect of k variables with 4n experiments, where 4n is the smaller multiple of four greater than k. In our case, a total of 12 experiments were performed to study the main effect of the factors and hence identify the significant ones out of the seven involved. Since only 8 experiments are necessary to determine the coefficients of the factors and the constant term of a linear model, we had 4 degrees of freedom, associated to the so called “dummy factors”; they are not real factors, obviously related to non-significant effects and therefore used to evaluate the significance of the real variables studied. The experiments were performed in random order to avoid systematic errors due to time effect. The same pool sample, described in the previous paragraph, was used in all experiments. After analysing the extracts derived from the different experiments, the

responses were used to build linear models and to find the significant factors. The open-source software "R" (R core team, 2014), provided with a chemometric "toolbox", was used for computation of the models by multiple linear regression (MLR) and for statistical evaluations.

Three factors out of seven were identified as significant by the analysis of the results of the screening design. Therefore, they were further studied, while the others were set at the most convenient value from a practical point of view. The Box-Behnken design [74] was selected as response surface design and used to build quadratic response models. In this design each variable is explored at three levels, coded as -1, 0 and +1. The significant variables and the levels investigated in this new design are shown in Table 28. The number of experiments (N) of a Box-Behnken design is given by the following formula:

$$N = 2k\,(k-1) + n_c$$

Where k is the number of factors and n_c is the number of replicates of the central point of the design. Hence, a total of 15 experiments (3 replicates of the central point) were carried out to evaluate linear, quadratic and interaction terms in the model. Analogously to the previous design, the experiments were performed in random order and the same pool sample was used in all tests.

Table 28: Factors investigated in the Box-Behnken design and selected levels.

Factor number	Factor	-1 level	0 level	+1 level
1	V_{solv}/m_{sample} (mL/mg)	0.025	0.0375	0.05
2	PSA sorbent (mg)	0	25	50
3	Florisil sorbent (mg)	0	50	100

*clean-up performed on 2 mL of extract

After the analysis of the extracts by HPLC-MS/MS, the responses associated to both recovery and matrix effect were used to build the single quadratic models for each response evaluated. The open-source software "R" was used for computation of the models by MLR and for statistical evaluations. In particular, analysis of variance (ANOVA) was performed to test the statistical significance of the regression models. The significant models, visualized by mean of response surfaces and isoresponse plots, were used to identify the optimal conditions for attaining best recovery and matrix effect for the considered analytes. Since for DAID and GEN best results were found by analysing the extract without any clean-up, some dilution tests were performed. The value which lead to minimum ion suppression was chosen for the optimized procedure.

Final extraction procedure

The optimized QuEChERS procedure for the pre-treatment of soy-burgers is here described. 200 mg of freeze-dried soy burger was weighted in a 15 mL plastic centrifuge tube, 10 mL of ACN:H_2O (50:50, v/v) was added and manually shaken for 3 min. Then, 4 g of $MgSO_4$ and 1 g of NaCl were added, vigorously shaken for 1 min and centrifuged at 5000 rpm for 5 min; this step allowed the phase separation of ACN and water, thanks to the salting out effect. At this stage, two aliquots of the organic layer were picked up and treated differently. The first aliquot, of 1 mL, was filtered through a 0.2 µm PTFE filter (Whatman, Maidstone, UK), diluted 1:160 in MeOH, and analyzed by HPLC-MS/MS. This sample was used for the determination of DAID and GEN. The second aliquot, of 2 mL, was subjected to the clean-up step: dispersive SPE was performed with 300 mg of $MgSO_4$, 37 mg of PSA sorbent and 60 mg of Florisil sorbent, again shaking vigorously for 1 min. The tube was centrifuged at 5000 rpm for 5 min, the supernatant collected, and an aliquot of 1.4 mL was dried under N_2. After reconstituting with the same volume of MeOH, the extract was filtered through a 0.2 µm PTFE filter and analyzed by HPLC-MS/MS. This sample was used for the determination of COUM, FORM and BIOCH.

6.3 Results and Discussion

In order to attain accurate quantitation of the five phytoestrogens in the complex matrix of soy-based burgers, a careful optimization of the extraction procedure was obtained thanks to the multivariate approach of experimental design. The technique of choice, among the ones tested, was QuEChERS [61], because of the ease of application, efficiency, rapidness and customizability. Many variables are involved in this sample pre-treatment and the ones which could potentially influence recovery and matrix effect of the five analytes were considered for the optimization of the process. The starting point for optimization was the method previously used in our lab [113]. The amount of salts used for phase separation was kept constant (4 g of $MgSO_4$ and 1g of NaCl) since preliminary tests demonstrated efficient phase separation by the addition of these amounts. The quantity of $MgSO_4$ used during clean-up (300 mg for 2 mL of extract) was constant as well, since it was necessary to eliminate residual water and was supposed not to affect recovery or matrix effect of the analytes. On the other hand, the variables considered to have a possible effect were volume of solvent mix/sample mass ratio (V_{solv}/m_{sample}), extraction time, PSA clean up sorbent, C18 clean up sorbent, Florisil clean up sorbent, clean up time and shaking mode. To evaluate recovery, the responses considered were the peak areas normalized to the sample mass extracted in each experiment; concerning matrix effect (ME), it was determined by analysing the extracts spiked with a proper amount of the analytes

(solution A) and comparing the signal with the corresponding pure standard in MeOH (solution P). The formula used was the following:

$$ME\ (\%) = \ 100 * \frac{PA_A - PA_{NS}}{PA_P}$$

where $PA_{A,}$ PA_{NS} and PA_P are the peak areas obtained by analyzing solution A, extracts without spike (NS) and the standard solution P, respectively [87].

Two sequential experimental designs were performed to first screen the important variables and then to model quadratic responses, in order to find the optimum of the extraction/purification method.

Results of the screening design

When performing a screening study, it is fundamental to select a proper experimental domain of the variables; it has to be large enough to detect a possible influence on the response, but at the same time the effect of a factor should not cover the others. The domain of the factors screened by the Plackett-Burman design (indicated in Table 27) was chosen taking into account some preliminary tests and literature data, as well as practical considerations [48,50,114]. As for the V_{solv}/m_{sample} ratio, it was chosen by fixing the volume of solvent used at 10 mL and selecting two mass values that allowed to detect all the analytes without excessive matrix effect, considering previous analyses performed in our laboratory. Concerning the levels of the factors "shaking time", times commonly used in QuEChERS extractions were taken as central values [48,50] and the +1 and -1 levels were symmetrically set, remaining in a reasonable range. To study the effect of the clean-up sorbents, they were treated as semi-quantitative factors, since the -1 level corresponded to the absence of the sorbent, while the +1 level was chosen on the basis of previous studies on other food matrices [50,61].

In order to simplify the evaluation of the 10 responses considered, we decided to perform a principal component analysis (PCA) on the responses, to search a correlation among the five analytes and therefore use a cumulative response to compute the models. As for recovery, the PCA highlighted a strong correlation among DAID and GEN peak areas, and FORM and COUM peak areas, respectively (as shown in the loading plot in Fig. 24); moreover, the first two principal components (PC1 and PC2) enclosed the 52.28% and 39.76% of the total variance of the data.

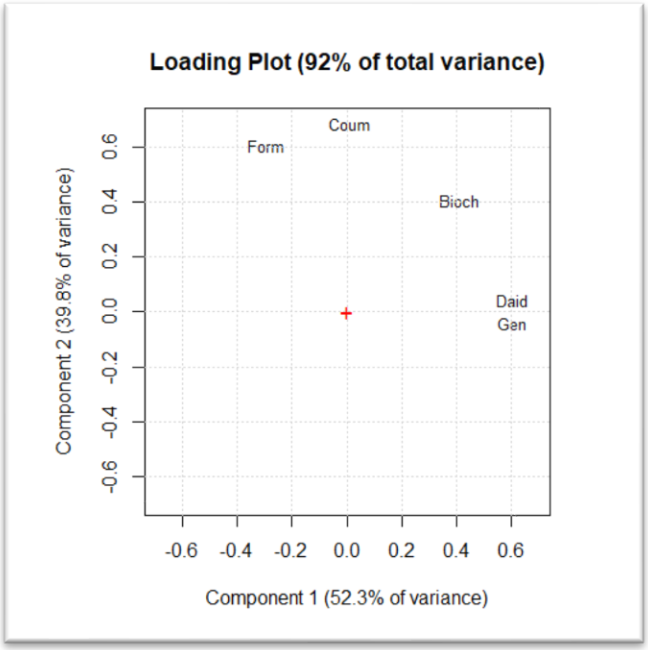


Figure 30: responses of the Plackett-Burman design: loading plot of the PCA performed on peak areas of the analytes; variables close in space are considered positively correlated.

Therefore, the scores on PC1 and PC2 were considered as two cumulative responses to be used to compute the linear models for recovery. The regression models obtained were characterized by high R^2_{adj}, of 0.9881 and 0.8178 for scores on PC1 and PC2, respectively. In the first case, the V_{solv}/m_{sample} ratio, the PSA clean-up sorbent and the C18 clean-up sorbent were significant at the p=0.05 confidence level; in the second case, the V_{solv}/m_{sample} ratio and the Florisil sorbent were identified as significant. Since correlation among peak areas was high, a further model based on the sum of the five peak areas, subjected to autoscaling was computed. By doing so, all data had the same weight with respect to the total, avoiding to give more importance to the components with larger peak areas. The results of the MLR were satisfactory, with a percentage of explained variance of the model equal to 86.77% (R^2_{adj}=0.8677). The coefficients which were found to be statistically significant at the p=0.05 confidence level, were the V_{solv}/m_{sample} ratio, the PSA clean-up sorbent and the Florisil clean-up sorbent. Fig. 31 graphically represents the significance of the factors with respect to experimental error (number of the factors defined in Table 27).

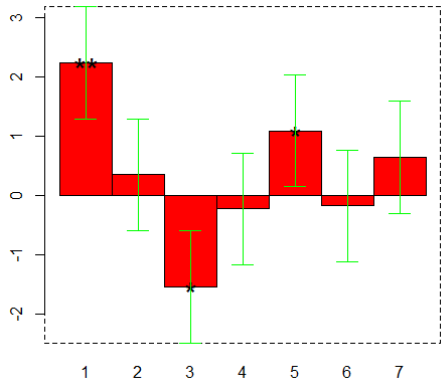


Figure 31: Graphical representation of the statistical significance of the 7 factors for recovery response (sum of the autoscaled areas). The asterisks indicate significance at the p=0.05 level or below.

As for the ME%, the analytes were considered as two different groups; while DAID and GEN were determined in a 130-fold diluted extract, COUM, FORM and BIOCH were determined in the non-diluted extract, hence we can consider that we had two completely different matrices. High correlation was found between matrix effect percent values (ME%) of DAID and GEN and among ME% of COUM, FORM and BIOCH; therefore, once again the sum of autoscaled values were used as responses to compute the models. The model obtained for the sum of DAID and GEN ME% showed a low percentage of explained variance (43.83%) and no coefficients were found to be significant at the p=0.05 confidence level. This result suggested that the matrix effect observed in the diluted extract was independent from the values of the variables studied. In fact, in all experiments ME% were around 80-90% for DAID and GE, with no substantial difference among the 12 experiments. It is reasonable that the dilution operated caused a strong decrease of the ion suppression, independently from the clean-up step. On the other hand, the MLR applied to the sum of COUM, FORM and BIOCH ME% gave very good results, with an 86.6% of variance explained by the model and the identification of three significant factors at the p=0.05 confidence level: the V_{solv}/m_{sample} ratio, the PSA clean-up sorbent and the Florisil clean-up sorbent.

The R^2_{adj} values, coefficients and confidence levels (p values) of all the computed models are reported in Table 29.

Table 29: summary of the models obtained by performance of the Plackett-Burman design, indicating the model fitting (R^2adjusted values) and the coefficients significance.

Model response	**Model R^2 adj**	**Coefficients**	**p value (significance level)**
PC1 scores (PCA on peak areas)	0.9881	b_1 (V_{solv}/m_{sample} ratio)= 0.2927	0.0045
		b_3 (PSA)= -1.4949	0.0001
		b_4 (C18)= -0.1679	0.0301
PC2 scores (PCA on peak areas)	0.8178	b_1 (V_{solv}/m_{sample} ratio)= 1.102	0.0032
		b_5 (Florisil)= 0.525	0.0390
Sum of peak areas (autoscaled)	0.8677	b_1 (V_{solv}/m_{sample} ratio)= 2.233	0.0028
		b_3 (PSA)= -1.54	0.0106
		b_5 (Florisil)= 1.087	0.0331
Sum of DAID and GEN ME% (autoscaled)	0.4383	No significant terms	/
Sum of COUM, FORM and BIOCH ME% (autoscaled)	0.8660	b_1 (V_{solv}/m_{sample} ratio)= 1.173	0.0185
		b_3 (PSA)= 2.167	0.0021
		b_5 (Florisil)= 0.9085	0.0410

The same three variables (V_{solv}/m_{sample} ratio, PSA clean-up sorbent and florisil clean-up sorbent) were identified as statistically significant for most models (recovery of the five analytes and matrix effects of COUM, FORM and BIOCH); the only exception was the model for the PC1 scores of peak areas: in this model also C18 was identified as significant, but the value of the coefficient was lower than the others. When performing response surface designs, exploring more than 3 variables considerably increases the number of required experiments while making data interpretation more difficult. For all these reasons, we decided not to consider this variable for the subsequent design. Hence, the optimization by response surface methodology was performed by selecting V_{solv}/m_{sample} ratio, PSA clean-up sorbent and florisil clean-up sorbent as factors.

Results of the response surface design

Response surface methodology is based on the computation of quadratic empirical models which are approximations of the reality in a well-defined experimental domain. Quadratic models are obtained by different kinds of experimental designs. The Box-Behnken design was chosen for this work, because of its favourable statistical characteristics; it is rotatable and defined as quasi-orthogonal. The rotatability guarantees a symmetrical variance function, which is used for the calculation of the

confidence interval in prediction; this feature permits to get better estimates of the optimum [115]. As for quasi-orthogonality, it means that the covariance among the coefficients is zero for most terms and very close to zero for the others, thus obtaining a better estimation of the coefficients.

The choice of the new experimental domain to investigate was carefully evaluated and the new levels are reported in Table 28. The limits of the domain for the variable V_{solv}/m_{sample} ratio, chosen in the previous design, was maintained: the -1 and +1 levels corresponded to 10 mL of solvent mix for 400 mg of sample and 10 mL of solvent mix for 200 mg of sample, respectively. These levels allowed to avoid too low sensitivity for the less concentrated analytes as well as excessive ion suppression. From the results of the screening design, it emerged that PSA had a strongly negative effect on the recovery of DAID and GEN. Therefore, higher amounts, with respect to the ones used in the Plackett.Burman design, were not desirable. The +1 level thus corresponded to 50 mg of PSA for 2 mL of extract while the -1 level was kept at 0 mg of sorbent. Finally, the domain for Florisil was slightly enlarged: since its effect was positive both for recovery and matrix effect, a larger mass of sorbent was selected as the +1 level, namely 100 mg for 2 mL of extract.

The experimental matrix, indicating the conditions of each experiment of the design is shown in Table 30. The general second order model that we wanted to compute for each response was the following:

$$Y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{1 \le i < j}^{k} b_{ij} x_i x_j + \sum_{i=1}^{k} b_{ii} {x_i}^2$$

where Y is the response, k is the number of variables, x_i are the variables, b_0 is the constant term, b_i, b_{ij}, and b_{ii} are the coefficients of the first order terms, of the interactions and of the second order terms, respectively.

Table 30: Experimental matrix of the Box-Behnken design.

	V_{solv}/m_{sample} (mL/mg)	PSA sorbent (mg)	Florisil sorbent (mg)
Exp 1	0.025	0	50
Exp 2	0.05	0	50
Exp 3	0.025	50	50
Exp 4	0.05	50	50
Exp 5	0.025	25	0
Exp 6	0.05	25	0
Exp 7	0.025	25	100
Exp 8	0.05	25	100
Exp 9	0.0375	0	0
Exp 10	0.0375	0	100
Exp 11	0.0375	50	0
Exp 12	0.0375	50	100
Exp 13	0.0375	25	50
Exp 14	0.0375	25	50
Exp 15	0.0375	25	50

The purpose of response surface methodology is to predict responses in points of the domain which are not directly investigated, thus finding optimal conditions which has to be empirically verified [73]; therefore, to obtain predictions easily comparable with experimental values, the models computed at this stage were based on single responses: to evaluate recovery, the peak areas normalized to the mass of sample were considered, while matrix effects were estimated as already described. Multiple linear regression was applied to these data, and empirical second order models were obtained.

ANOVA was performed for statistical evaluation of the mathematical models, thus gaining information on significance of the factors' coefficients as well as the overall regression model and possible lack of fit [116]. Table 31 summarizes the results of ANOVA for the computed models which resulted statistically meaningful, as well as the reduced functions, including only the significant coefficients. In the reduced models the non-significant terms are simply not shown, but the model is not re-computed. In fact, re-computation after eliminating the non-significant terms should be avoided, since this action would affect also the computation of the significant ones and could cause overfitting of the model.

Table 31: ANOVA results for the computed models.

Response	Reduced model	Coefficients significance (p value)	Regression significance (p value)	Lack of fit significance (p value)*	R^2	R^2 adj
GEN recovery	$y = 19439-5526.9\ x_2$	x_2: p= 0.0006	0.0233	0.6164	0.9256	0.7917
DAID recovery	$y = 43587-7261.1\ x_2$	x_2: p= 0.0004	0.0111	0.5660	0.9457	0.8481
COUM recovery	$y = 673.667+82x_1-149.5x_2x_3-119.08x_2^2$	x_1: p= 0.0275 x_2x_3: p= 0.0107 x_2^2: p= 0.0288	0.0046	0.5923	0.8938	0.7026
COUM ME%	$y =56+5.375x_1+9.625x_2-5x_2x_3-5.875x_3^2$	x_1: p= 0.0136 x_2: p= 0.0011 x_2x_3: p= 0.0579 x_3^2: p= 0.0395	0.0149	0.3061	0.9386	0.8281
FORM ME%	$y =53+3.25x_1+3.75x_2+2.25x_3-4x_2^2$	x_1: p= 0.0096 x_2: p= 0.0054 x_3: p= 0.0372 x_3^2: p= 0.0192	0.0214	0.4314	0.9283	0.7992

*the lack of fit must be non-significant, therefore p values>0.05 are expected for the 0.05 confidence level.

Unfortunately, not all the models satisfied the ANOVA significance requirements; the model for recovery of FORM had a significant lack of fit and low R^2_{adj} value; the models for recovery and matrix effect of BIOCH were characterized by rather low R^2_{adj} value and no significant terms. Nevertheless, the other models were used to find the optimal conditions. The correlation observed among both peak areas and matrix effects of COUM, FORM and BIOCH made it likely that the best conditions identified by the meaningful models could be adequate to optimize all responses.

The models which resulted statistically satisfactory were further validated by performing three experiments external to the set used for modelling. These experiments were chosen in the central point of the domain, since the leverage function (used to calculate the confidence interval of a prediction) usually has a minimum in this point. If the model is validated in the central point, it is likely that is valid also in the rest of the domain. Table 32 indicates the predicted and observed values of peak areas and matrix effects, with the corresponding confidence interval (0.05 confidence level); all the considered models resulted validated at the 0.05 confidence level in the centre of the domain.

Table 32: validation of the models in the central point of the domain.

Response	Predicted value	Observed value
DAID recovery (peak area*)	43600 ± 5400	39800 ± 2900
GEN recovery (peak area)	19400 ± 6400	17400 ± 1700
COUM recovery (peak area)	670 ± 10	640 ± 110
COUM ME% **	56 ± 8	59 ± 5
FORM ME%	53 ± 6	53 ± 5

*peak area expressed as arbitrary units

**matrix effect expressed as percent values accordingly to the formula presented in section 6.3

Three-dimensional response surfaces and isoresponse plots were obtained by the R software and used to identify the variables settings corresponding to regions of maximum responses. By these graphics we can represent the response as a function of two variables, while the third must be set at a constant value. Fig. 32 represents the response surfaces and isoresponse plots for GEN, DAID and COUM recovery, where the significant variables were selected as x and y axes. Figure 33 represents the plots for COUM ME% and FORM ME%; here, the selected variables were PSA and Florisil, while the V_{solv}/m_{sample} ratio was set constant at the +1 level, since the influence of this factor was positive (therefore maximum response is expected to be at the +1 level).

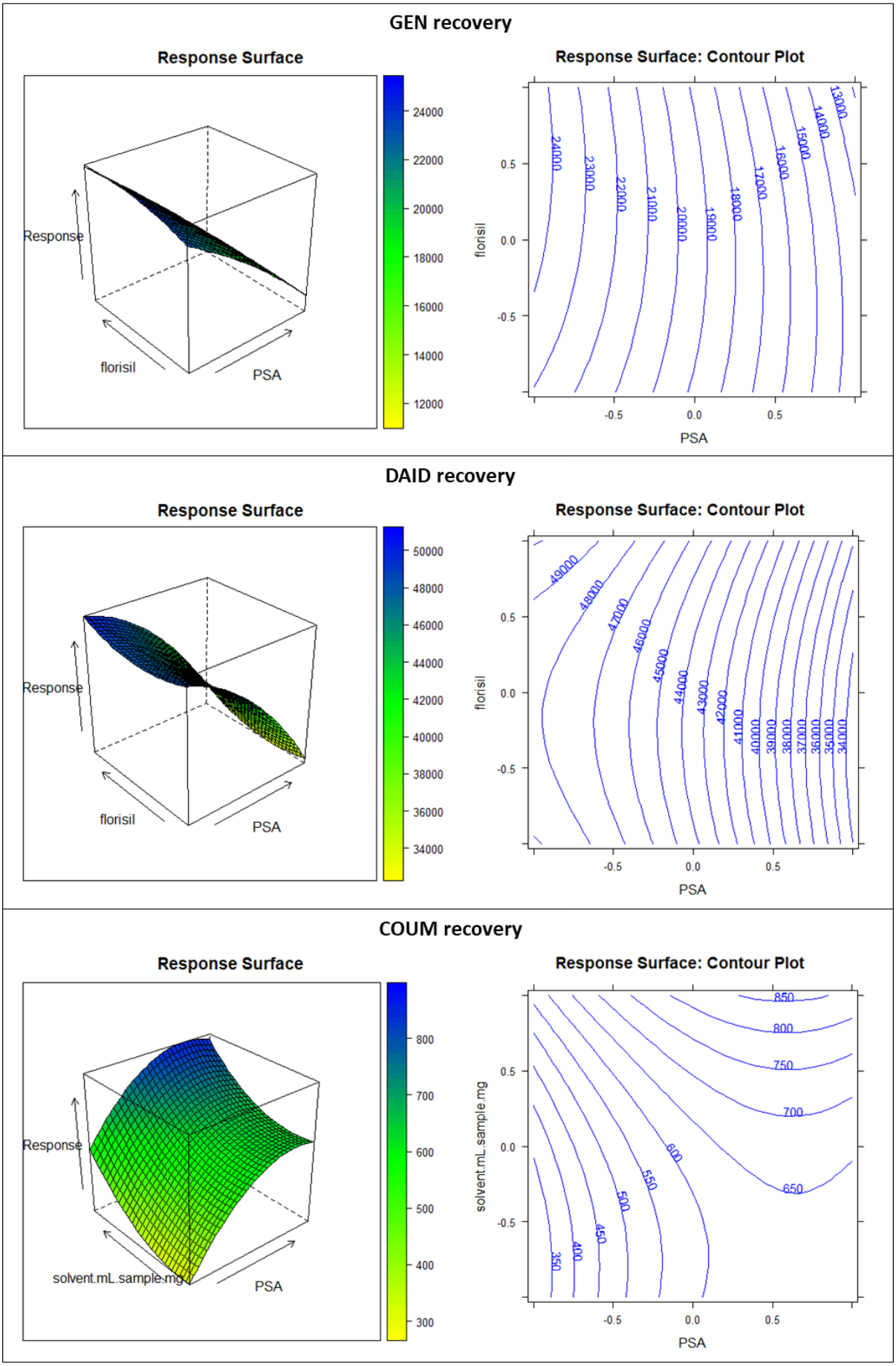


Figure 32: Response surfaces and isoresponse plots of the second order models for recovery.

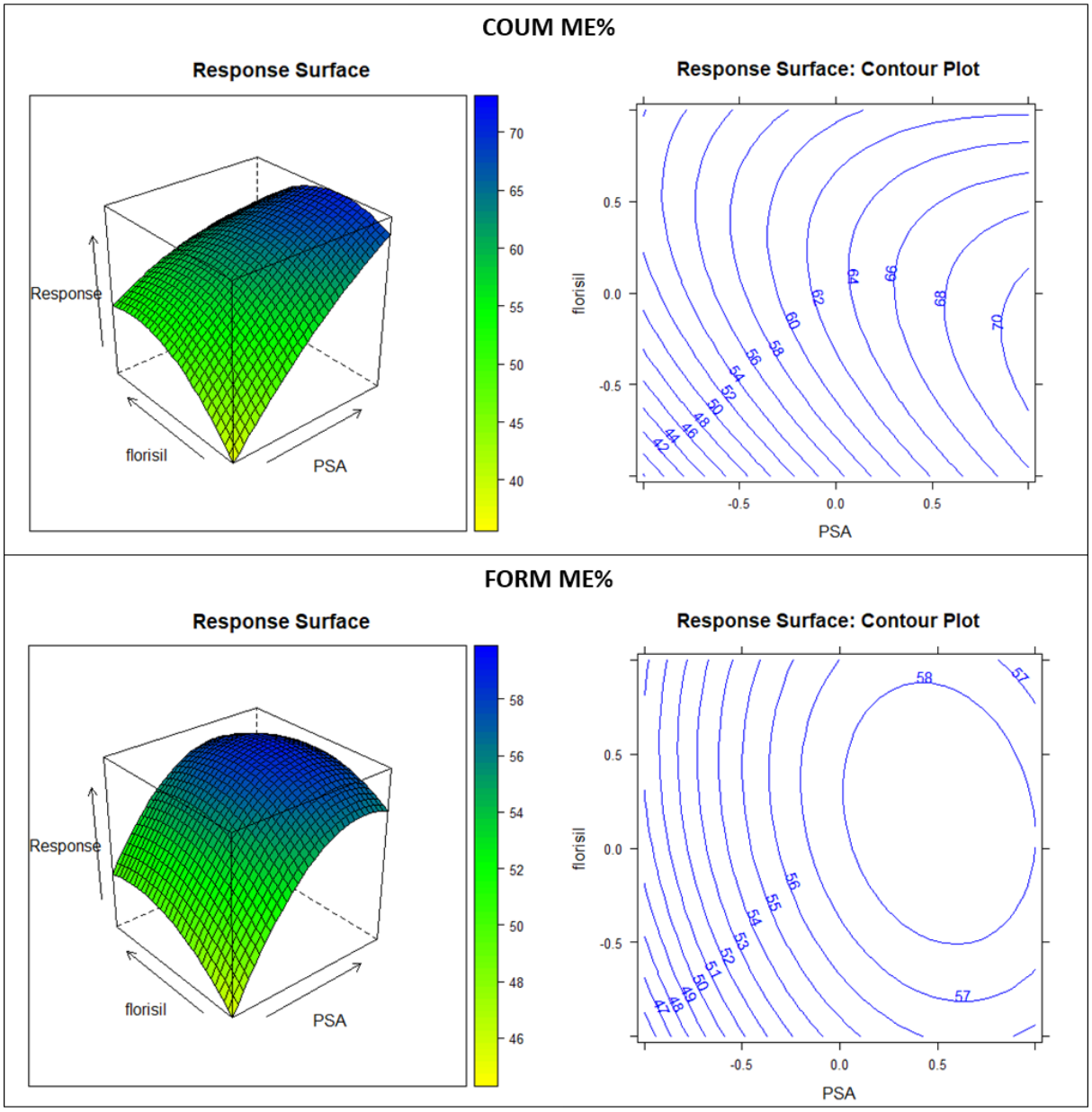


Figure 33:Response surfaces and isoresponse plots of the second order models for ME%.

Selection of the optimal conditions

Some evaluations based on the ANOVA results, as well as visual inspection of response surfaces, were used to find the "optimum". When searching for the optimal conditions after performing a Box Behnken design, we have to keep in mind that the combinations of all extreme values (all -1 values, for instance) of the variables are not investigated by the design (the model does not include these points). Therefore, if the mathematical optimum is in one of these combinations, it should not be considered, since the model does not represent this external part of the domain. Alternative "acceptable" regions should be evaluated.

The models for GEN and DAID recovery were linear, being the PSA sorbent amount the only significant factor; this indicates that the sorbent probably binds the two analytes and must be avoided

for their determination. In fact, by simply looking at the results of the experiments, peak areas of experiments in which PSA were used were at least half the ones in which this sorbent was not used. Moreover, the screening design demonstrated that GEN and DAID matrix effect was not influenced by any of the studied factors. Therefore, clean-up was not useful, but deleterious for the determination of GEN and DAID, and we decided to quantify these two analytes by analysing an aliquot of the extract prior to clean-up. Since dilution was an important aspect to consider, different dilution ratios were tested. The ACN fraction obtained after the extraction and phase separation steps of the QuEChERS procedure, was subjected to 80-fold, 100-fold, 120-fold, 140-fold and 160-fold dilutions in MeOH. As expected, best results were obtained with the maximum dilution; higher dilution ratios were not tested to guarantee the analytes detection in real samples.

Concerning the other models, namely COUM recovery, COUM ME% and FORM ME%, a list of candidate optimal points was drawn up, by looking at the response surfaces and isoresponse plots. For all models the best setting of the V_{solv}/m_{sample} ratio was the coded level +1. As for the other two variables, different candidate points were identified. Best COUM recovery was expected in a rather wide region, with PSA coded level in the range 0-0.5 and Florisil in the whole range; in fact, the only significant term related to Florisil was the interaction with PSA, resulting in a limited influence on the response. Maximum COUM ME% was expected at PSA coded level +1 and Florisil coded level 0. Finally, maximum FORM ME% was likely to be obtained at PSA coded level 0.5 and Florisil coded level 0.25. When searching for the "sweet spot", we have to keep in mind that the prediction as well as the variables are affected by errors; hence, it is more reliable to pinpoint a region or a list of best values for the studied variables. This assumption is even more important when multiple responses have to be considered for the optimum localization. The "multi-criteria decision making" approach of Pareto fronts was exploited to find the best compromise among the optimal conditions identified for the different responses [77,78]. A list of candidate predicted responses were plotted against one another, and the non-dominated point, which corresponded to acceptable values for all responses, was selected as the optimal set of conditions.

The final values selected as optimal were: 10 mL of solvent for 200 mg of burger sample, 37 mg of PSA sorbent and 60 mg of Florisil sorbent (for the clean-up of 2 mL of extract). When performing the optimal experiment as well as the treatment of real samples, a ±10% of these optimal values were considered fully acceptable. Three replicates of an experiment at the optimal conditions were performed. So far, recovery was evaluated by simply considering the peak areas of the substances, but in this case, a full recovery evaluation was carried out, by spiking samples before and after extraction, to actually obtain percent recoveries of the analytes. The optimal experiment gave

satisfactory results, in good accordance with model predictions; these results are shown in Table 33, jointly with the precision of the optimal QuEChERS procedure (6 replicate extractions considered).

Table 33: Results obtained by performing the optimal experiment.

Compound	REC % (n=3)	ME % (n=3)	Precision (RSD%, n=6)
DAID	102 ± 4	80 ± 6	2.2
GEN	104 ± 3	93 ± 7	1.8
COUM	101 ± 3	77 ± 2	2.4
FORM	104 ± 4	76 ± 3	3.2
BIOCH	97 ± 6	60 ± 2	3.2

Excellent recoveries and precision were reached, as well as reasonable matrix effects for all compounds. In fact, a 20% of ion suppression or enhancement are considered acceptable when performing quantitation. The only analyte with ME% out of the acceptable range was BIOCH. By looking at the qualitative MRM transition of this analyte, an interferent specie was detected at a real close retention time; this could account for the difficulties found to optimize its matrix effect.

As performed with the central point of the domain, the responses observed in the optimal experiment were compared with those predicted by the models. Table 34 indicates the predicted and observed values of peak areas and matrix effects, with the corresponding confidence interval (0.05 confidence level); even though not all the predicted and observed values were perfectly concordant, the differences found were acceptable or even better results than expected were attained.

Table 34: comparison between predicted and observed values for the optimal experiment.

Response	Predicted value	Observed value
DAID recovery (peak area*)	31800 ± 4000	22800 ± 1900
GEN recovery (peak area)	15600 ± 4500	10000 ± 1000
COUM recovery (peak area)	780 ± 20	800 ± 100
COUM ME% **	67 ± 9	77 ± 6
FORM ME%	58 ± 6	76 ± 8

*peak area expressed as arbitrary units

**matrix effect expressed as percent values accordingly to the formula presented in section 3

6.4 Conclusions

When dealing with accurate quantitation in complex matrices, it is fundamental to optimize the sample pre-treatment, in order to reach maximum recovery and minimize the influence of matrix interferents, especially if LC-ESI-MS is used as analytical technique. Soy burgers are a mix of soy, vegetables and cereals, thus constituting a composite matrix. Even if a small number of analytes is determined, optimizing both recovery and matrix effect in this kind of sample is not a trivial task. In our case, ten were the responses to be optimized (recovery and ME% for DAID, GEN, COUM, FORM and BIOCH). The QuEChERS technique is a fast and easy way to obtain efficient extraction and clean-up, but since several variables are involved, an accurate study is necessary to maximize the performances. The OVAT approach is not suitable if we want to find a real optimum, understanding the effects of the variables and their interactions in a certain domain. Thanks to the multivariate approach of experimental design a rational plan of the experiments was carried out: a first screening design (12 experiments) allowed to identify three significant factors out of seven; a subsequent response surface design (15 experiments) served to model the responses as a second order function of the considered variables. The models obtained were subjected to ANOVA and validated in different points of the experimental domain. The optimal conditions were identified by looking at the response surfaces and using the Pareto front approach (for multicriteria decisions). Quantitative recoveries (97-104%) as well as satisfactory matrix effects (60-93%) were obtained for all analytes, after performing a total of 33 experiments, including validation and performance of the optimum.

Chapter 7

Determination of phytoestrogens in different soy burgers from the Italian market

7.1 Introduction

A wide range of soy-based products is spreading in the European market in the latest years. They are vegetarian or vegan preparations which are usually proposed as alternative to products of animal origin. Alongside soymilk, soy yogurts and tofu, presented as alternatives to dairy products, some vegetable recipes are proposed in form of burgers, cutlets and sausages. Soy is usually one of the main ingredients of these goods, since the high protein content makes it an ideal "meat substitute". Very few papers deal with quantitation of phytoestrogens in soy burgers, especially because of their relatively new appearance on the market, and commonly they are included in a general group of "soy foodstuff". Only one work was found on products from Italy [112], but the studied substances were the most concentrated isoflavones found in soy (daidzein, genistein and their glycosylated derivatives), and no optimization was performed on sample preparation. Indeed, daidzein and genistein levels were quite low if compared to those detected in the previous parts of this doctorate

work, as well as other literature data [117]. Moreover, there is a complete lack of data about the fate of phytoestrogens in processed food. In fact, while soy milk and other soy derivatives are commonly consumed without any preparation, soy burgers must be cooked, following the suggestions given on the boxes. The effect of cooking on phytoestrogens content of these products has never been investigated so far. For all these reason, we decided to apply the optimized QuEChERS procedure to analyse a quite large group of soy-burgers, purchased in different Italian supermarkets, both raw and cooked, to investigate phytoestrogens content before and after food preparation.

7.2 Materials and methods

Chemicals

The phytoestrogens standards (DAID, GEN, FORM, BIOCH and COUM) were the same used in the previous part of the work, purchased from Sigma Aldrich (St. Louis, MO, USA). The materials used for QuEChERS procedure, namely magnesium sulfate, sodium chloride, Florisil and Primary Secondary Ammine (PSA) loose sorbents, were the same described in chapter 6.
Methanol (MeOH) and acetonitrile (ACN) were from VWR Chemicals (Fontenay-sous-Bois, France), while formic acid was obtained from Sigma Aldrich (Steinheim, Germany). All solvents were of chromatographic grade. Ultra-pure water was obtained from a Millipore Q-Gard system equipped with a Millipak 0,22 µm filter (Millipore, Watford, Hertfordshire, UK).

Instrumental analysis

The method used for the determination of the five phytoestrogens was the same described in chapter 5. The analyses were performed using the instrument already mentioned before: Agilent 1200 SL high performance liquid chromatograph coupled to an Agilent 6430 triple quadrupole mass spectrometer with an ESI ion source. The chromatographic column used was the pentafluorophenyl (PFP) column and chromatographic separation was achieved within 9 min with the conditions described in chapter 5.
The mass spectrometric conditions used were the same optimized and discussed in chapter 4 (MRM parameters reported in Table 16). The MRM transitions used for quantitative purposes and the retention times of the analytes were reported in Table 21 (chapter 5).

Calibration

The calibration performed for the study in chapter 5, involved the use of curves in a linearity range from 0.5 to 200 µg L^{-1}. However, the less concentrated analytes were below the lowest point of the curve, while DAID and GEN concentration in the diluted extract never exceeded the 20 µg L^{-1}. For this reason, we decided to slightly change the limits of the calibration curves, starting from the LOQ of the less sensitive analytes (0.2 µg L^{-1} for GEN and BIOCH) and ending at 100 µg L^{-1}, to be sure that all new samples had a GEN and DAID concentration inside the calibration curve. Linearity was verified inside this new concentration range, as well as determination coefficient and instrumental precision (intra-day and inter-days assays). LODs and LOQs were estimated again, since the optimized clean-up led to slightly lower background noise, with consequent improvement of sensitivity.

Samples and sample treatment

Twelve types of soy-based burgers were purchased in Italian drugstores (Fig. 34). For each type, a burger was cooked, following the manufacturer suggestions; generally, the indication was 1-3 minutes of microwave cooking. The raw and cooked samples were minced and freeze-dried for 24 hours. After that, they were further ground with a pestle to obtain a fine and homogenous powder. The freeze-dried powder was store at -18°C until extraction. The QuEChERS protocol optimized by experimental design was used for sample pre-treatment (description of the whole procedure in chapter 6, section 6.2). Both the non-diluted aliquot subjected to clean- up and the diluted aliquot (1:160, no clean-up) were filtered through a 0.2 µm PTFE filter and analyzed by HPLC-MS/MS.

Figure 34: picture of the twelve soy-based burgers purchased in different Italian drugstores.

Data analysis

The results of the analyses were elaborated by performing principal component analysis (PCA). The open source software "R", provided with a chemometric "toolbox", was employed to obtain PCA; loading plots, score plots and biplots were used for data interpretation.

7.3 Results and Discussion

Analytical performances

The main figures of merit of the instrumental method were evaluated again, since the limits of the calibration curves were changed. Moreover, the overall analytical method was different with respect to the one described in chapter 5. The linearity range, LODs and LOQs, intra-day and inter-days precision for the five phytoestrogens are summarized in Table 35.

Table 35: figures of merit of the instrumental method in the new concentration range selected.

Compound	R^2	Linearity (µg L^{-1})	LOD (µg L^{-1})	LOQ (µg L^{-1})	Intra-day Precision (RSD%)	Inter-days Precision (RSD%)
DAID	0.9943	0.2-100	0.02	0.05	2.0	11.9
GEN	0.9999	0.2-100	0.06	0.20	4.1	6.4
COUM	0.9988	0.2-100	0.01	0.03	4.0	8.9
FORM	0.9928	0.2-100	0.02	0.07	2.4	14.0
BIOCH	0.9999	0.2-100	0.06	0.20	2.4	7.8

The linearity was verified in the new range for all compounds, achieving good determination coefficient (R^2) for all calibration curves (>0.99). Except for FORM, which already had a rather low LOD, the sensitivity was improved for all analytes, with LODs and LOQs in the range of 0.01-0.06 µg L^{-1} and 0.03-0.2 µg L^{-1} respectively (evaluated with respect to the analyzed extracts). Intra-day and inter-day precision were assessed at all concentration levels of the calibration curve; the average values obtained, expressed as relative standard deviation (RSD), were in the range 2.0-4.1% and 6.4-14% respectively. All validation parameters were satisfactory in the new linearity range. Repeatability was slightly worse with respect to the other range; this is reasonable taking into account the shift of the calibration curve to lower concentrations. However, all values were fully acceptable, with intra-day and inter-day RSD generally below 4% and 14%, respectively.

Quantitation of commercial samples

In order to perform the quantitation on real samples, the lower limits for detection and quantitation of the analytes were calculated with respect to the mass of soy burger; the LODs and LOQs values are reported in Table 36.

Table 36: LODs and LOQs expressed as ng g^{-1} of soy-burger sample.

Compound	LOD (ng g^{-1})	LOQ (ng g^{-1})
DAID	0.4	1.3
GEN	1.5	5.0
COUM	0.2	0.8
FORM	0.5	1.7
BIOCH	1.5	5.0

To have a broad picture on the concentration levels of the considered analytes in commercial soy-burgers, a total of 12 fresh soy-burgers (not frozen) were purchased. In particular, all veggie burgers were bought in Ligurian drugstores, and well resemble the Italian large retailers. Table 37 shows the brands, percentage of soy and other main ingredients of the twelve analyzed samples.

Table 37: Main ingredients of the analyzed soy-burgers and sample brands.

Sample	Brand	Soy content (% w/w)	Vegetables	Cereals
1	Granarolo	20%	Zucchini, potatoes, leek	Wheat
2	Granarolo	34%	Tomato, olives, carrots, potatoes, celery	Wheat
3	Carrefour	19%	Carrots, onion, potatoes	Millet, corn
4	Sojasun	47%	Onion, tomato	/
5	Sojasun	41%	Tomato, olives	Wheat
6	Sojasun	44%	Asparagus, potatoes	Wheat
7	Valsoia	18%	Potatoes, peas, carrots	Corn, rice
8	Valsoia	19%	Spinach, carrots, onion,	Quinoa
9	Coop	40%	Onion, celery, carrots, tomato	Wheat
10	Coop	6%	Spinach, potatoes, onion	Wheat
11	Bottega Vegetale	72%*	Olives, spirulina algae	/
12	Carrefour	10%	Spinach, potatoes, onion	Spelt, rice, corn

* texturized soy proteins

As already mentioned, we wanted to verify if the cooking procedure alters the amount of phytoestrogens in the burgers, as to really estimate the intake when consuming these foods. Therefore, each sample was analyzed raw and after microwave cooking, by applying the QuEChERS procedure and LC-MS/MS analysis. Quantitation was performed by external calibration, taking into account the recovery and matrix effect previously evaluated for the used technique. Results are shown in Table 38 and express the content of phytoestrogens in the dry mass, (lyophilized burgers). All samples were extracted once, analyzed in triplicate and the RSD related to the procedural precision was used to express the uncertainty of the results.

Table 38: phytoestrogens content (µg or ng g^{-1} of dry weight) of the twelve analyzed burgers: R indicates the raw samples, while C indicates the cooked samples.

Sample	DAID	GEN	COUM	FORM	BIOCH
	µg g^{-1}		ng g^{-1}		
S1R	52± 1	44.8 ± 0.8	14.1 ± 0.3	9.5 ± 0.3	<LOQ
S1C	53 ± 1	45.6 ± 0.8	13.6 ± 0.3	8.0 ± 0.3	<LOQ
S2R	34.3 ± 0.7	32.7 ± 0.6	14.1 ± 0.3	6.1 ± 0.2	<LOQ
S2C	34.1 ± 0.7	34.1 ± 0.6	11.4 ± 0.3	7.2 ± 0.2	<LOQ
S3R	73 ± 2	62 ± 1	12.3 ± 0.3	7.0 ± 0.2	<LOQ
S3C	71 ± 2	61 ± 1	12.7 ± 0.3	7.1 ± 0.1	<LOQ
S4R	26.3 ± 0.6	32.0 ± 0.6	25.4 ± 0.6	14.5 ± 0.5	<LOQ
S4C	26.2 ± 0.6	33.7 ± 0.6	26.2 ± 0.6	14.4 ± 0.5	<LOQ
S5R	7.9 ± 0.2	15.9 ± 0.3	17.2 ± 0.4	3.2 ± 0.1	<LOQ
S5C	7.3 ± 0.2	15.7 ± 0.3	15.2 ± 0.4	3.9 ± 0.1	<LOQ
S6R	18.6 ± 0.4	25.8 ± 0.5	20.3 ± 0.5	7.9 ± 0.3	<LOQ
S6C	16.6 ± 0.4	25.0 ± 0.5	21.6 ± 0.5	7.2 ± 0.2	<LOQ
S7R	78 ± 2	65 ± 1	12.5 ± 0.3	40 ± 1	10.0 ± 0.3
S7C	78 ± 2	67 ± 1	10.3 ± 0.3	37 ± 1	9.5 ± 0.3
S8R	75 ± 2	63 ± 1	12.0 ± 0.3	37 ± 1	7.5 ± 0.2
S8C	85 ± 2	70 ± 1	11.0 ± 0.3	36 ± 1	9.4 ± 0.3
S9R	15.0 ± 0.3	22.0 ± 0.4	37.9 ± 0.9	31 ± 1	3.7 ± 0.1
S9C	14.4 ± 0.3	20.9 ± 0.4	35.8 ± 0.9	28.3 ± 0.9	2.0 ± 0.1
S10R	10.6 ± 0.2	18.3 ± 0.3	15.6 ± 0.4	12.9 ± 0.4	2.3 ± 0.1
S10C	11.5 ± 0.3	18.6 ± 0.3	17.1 ± 0.4	11.8 ± 0.4	4.4 ± 0.1
S11R	23.0 ± 0.5	28.5 ± 0.5	10.5 ± 0.3	8.9 ± 0.3	<LOQ
S11C	16.6 ± 0.4	22.9 ± 0.4	8.0 ± 0.2	5.7 ± 0.2	<LOQ
S12R	36.0 ± 0.8	37.9 ± 0.7	6.1 ± 0.2	<LOQ	<LOQ
S12C	35.8 ± 0.8	37.0 ± 0.7	7.5 ± 0.2	<LOQ	<LOQ

As already highlighted by the other analyses performed during this thesis, soy-food products are rich in DAID and GEN, while the other isoflavones and COUM are at very low concentration. In fact, in all the twelve samples DAID and GEN were at the tens of ppm level, FORM and COUM were at the ppb level (up to a maximum concentration of 40 ng g^{-1}) and BIOCH was under the LOQ for most samples, except for the number 7, 8, 9 and 10. Apparently, no significant difference was found between raw and cooked samples; the increase in temperature and denaturation processes which occur during cooking did not affected the phytoestrogens levels.

The DAID and GEN concentrations are in accordance with data present in the literature for the same or similar matrices (soy burgers and soy cutlets) [52,53,113]. Moreover, the obtained values are of the same order of magnitude of the mean values reported for veggie burgers by a USDA database of isoflavones in soy food [117]. Few data are present about the concentration of COUM, FORM and BIOCH in soy burgers; two papers reported that these analytes were below the method detection limits [118,119], higher than the ones reached by the present method.

Data analysis by chemometrics

When chemical data are made of several observations and variables, it is not always simple to interpret and understand the obtained results in a univariate way. Graphical representations are useful, but no more than three dimensions can be visualized. PCA is a powerful tool to obtain the maximum information from a set of data, by looking at them as a function of some latent variables (principal components), enclosing the majority of the data variability. PCA was performed on the results of the quantitative analysis. Over 99% of the variability was explained by the first 3 principal components. This is quite common when few variables are studied, and strong correlations are present. Fig. 35 shows the loading plot, which clearly indicates high correlation between DAID and GEN (variables overlapped in the plot), but also between BIOCH and FORM (variables really close in the plot).

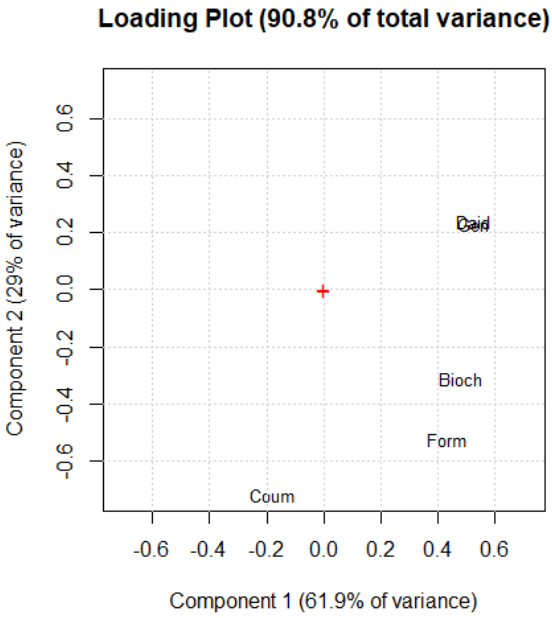


Figure 35: Loading plot of the PCA performed on the results of the soy-burgers analysis.

DAID, GEN, FORM and BIOCH all had a positive loading with respect to PC1, which represents the 61.9% of the total variance. COUM had a negative and small influence on PC1 computation, while was characterized by a large negative loading on PC2 (explaining the 29% of variability). By looking at the scores plot, some considerations can be made on the basis of different categories identified in the sample group. First of all, no separation is evident among raw and cooked samples, as shown in the 3-D score plot in Fig. 36 (a); this confirmed that the slight differences in phytoestrogens concentration of raw and cooked samples are mainly due to casual errors.

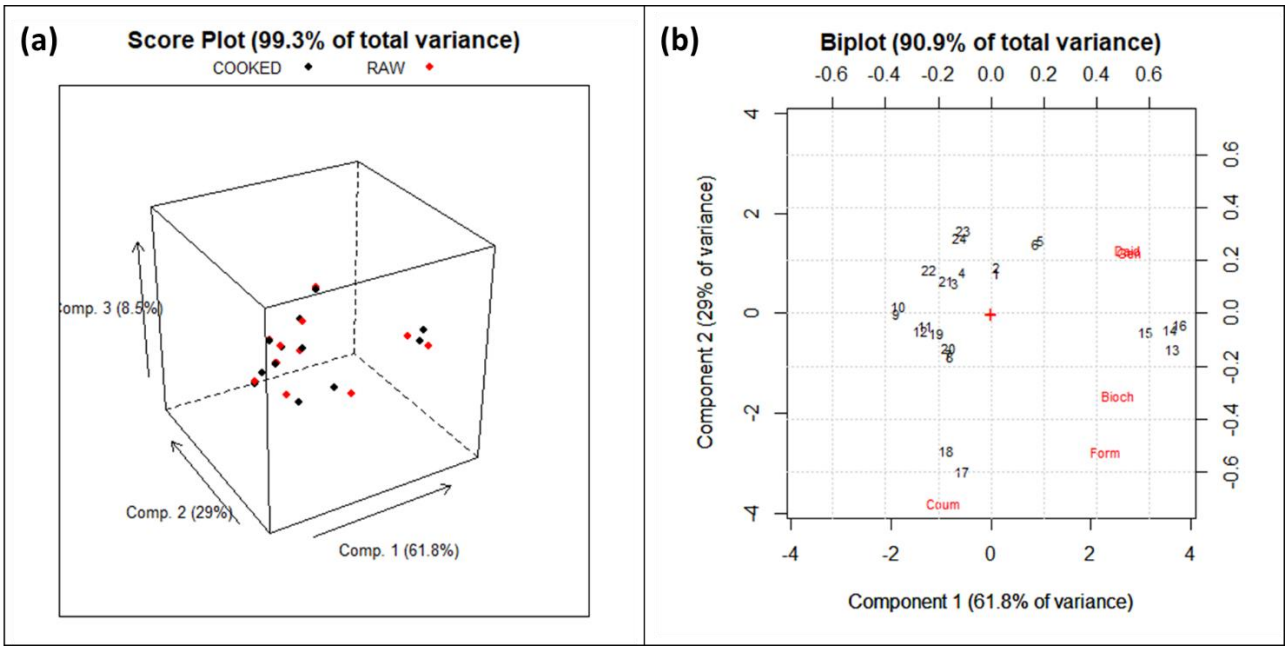


Figure 36: (a) 3-D score plot representing the samples categorized in "raw" and "cooked"; (b) biplot representing the original variables contribution and the scores of the samples altogether with respect to the PCs .

Some general evaluations can be made by the aid of the biplot (Fig. 36(b)); the samples are clearly divided into two groups, one with positive scores on PC1 (on the left of the plot), composed by sample 3, 7 and 8 (raw and cooked, number 5, 6, 13, 14, 15 and 16 in the figure), and one with negative scores on PC1 (on the right of the plot), composed by all the other samples. Sample 3, 7 and 8 are actually the ones with the higher content of DAID and GEN, as suggested by the proximity of the three samples to these original variables in the biplot. Sample 9 (raw and cooked, number 17 and 18 in the figure), which is far from all other samples and almost overlapped to COUM variable in the biplot, is the one with the highest content of COUM, but also FORM.

As mentioned, we tried to identify some patterns based on the “category” of the samples. The soy-burgers could be grouped on the basis of soy amount and of the commercial brand. Fig. 37 shows the 3-D score plot of the samples characterized by different soy content:

- Soy percentage <10% (samples 10, 12)
- Soy percentage 10-30 % (samples 1,3,7,8)
- Soy percentage 30-50% (samples 2, 4, 5, 6, 9)
- Soy percentage unknown (sample 11)

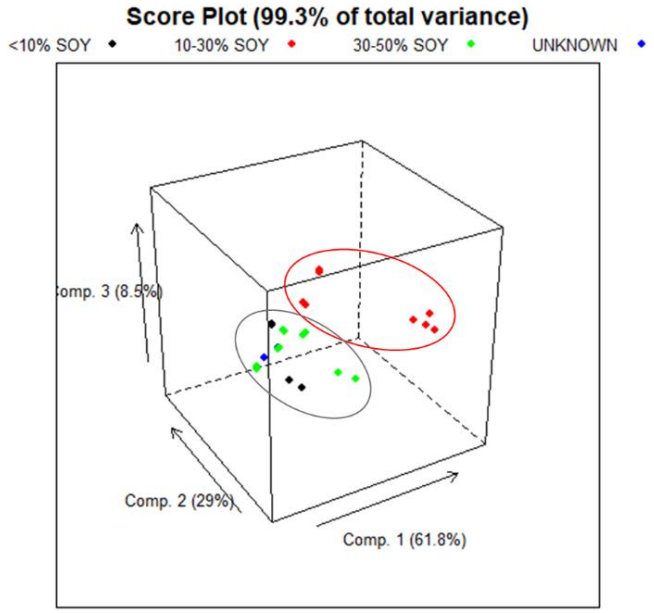


Figure 37: 3-D score plot representing the samples categorized by the different soy percentages.

Except for the samples with a soy percentage comprised between 10 and 30%, no particular pattern was observed for the other samples. This supports the hypothesis made so far that the percentage of soy is not the only factor influencing the total concentration of phytoestrogens, and no grouping can be made on this basis. On the other hand, interesting patterns were observed by looking at the brands of the different burgers. The 2-D and 3-D score plots, highlighting the products brands are shown in Fig. 38.

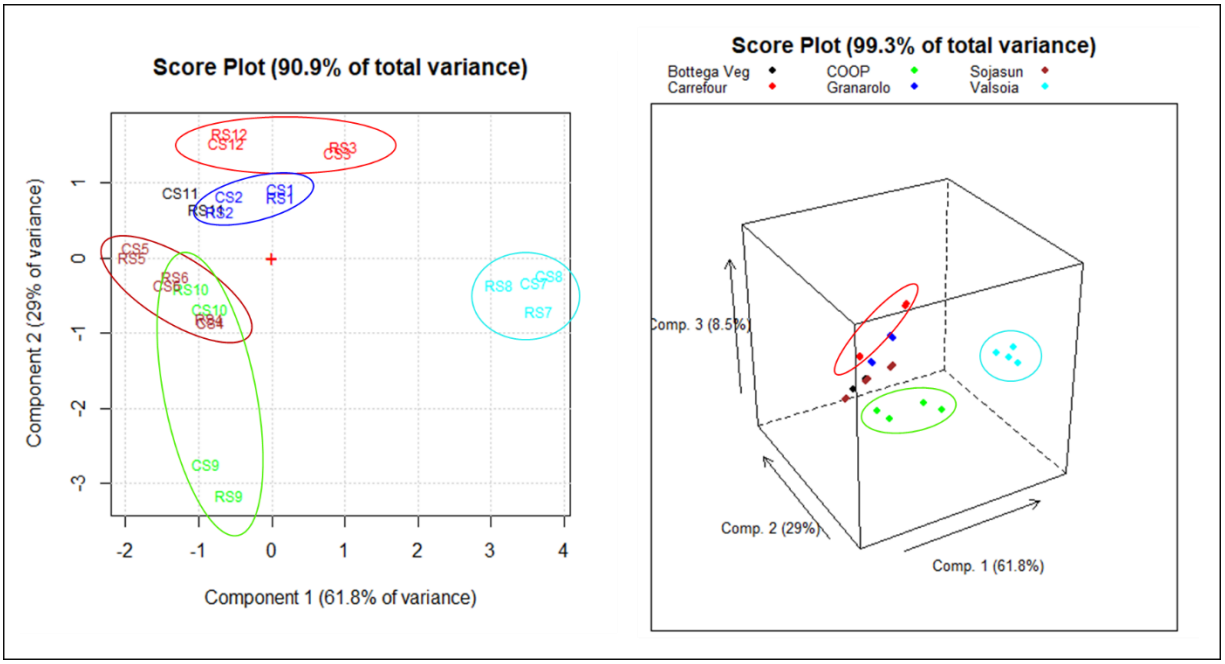


Figure 38: 2-D and 3-D score plots representing the samples categorized by the different commercial brands.

It is clear that small groups of samples belonging to the same manufacturer can be identified, because characterized by similar scores in the PCA. Therefore, it is reasonable that different soy varieties or batches used by the brands could account for different final concentrations of phytoestrogens. Clearly, a deeper study, based on the analysis of more samples from different lots of the same trademark would be necessary to confirm this trend.

7.4 Conclusions

The QuEChERS procedure carefully optimized, demonstrated to be fully applicable and suitable for the determination of the five phytoestrogens in commercial samples. The detection and quantitation even of the trace level phytoestrogens was successfully achieved. A representative sample of the soy-burgers present on the Italian market (12 different types) was purchased; both raw and cooked samples were analysed, for a total of 24 determinations. The QuEChERS procedure, combined with the fast and sensitive HPLC-MS/MS analysis allowed rapid and easy sample treatment, as well as accurate quantitation. Data elaboration, aided by chemometric tools (PCA), permitted to visualize the data and investigate the possible presence of sample grouping. No significant differences were found among raw and cooked samples. Not even the percentage of soy seemed to define particular groups. On the other hand, it was possible to identify some patterns due to the brands of the analysed samples, hence maybe due to different soy variety employed in burger preparation.

Chapter 8

Multivariate optimization of innovative pre-treatment strategies: an environmental application

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8.1 Introduction

Alongside the main work of the thesis, another topic was treated, in the framework of analytical method development and optimization. In particular, the work here described dealt with the multivariate optimization of the pre-treatment of environmental samples. The aim was to develop an innovative method for the determination of polycyclic aromatic hydrocarbons (PAHs) in sea water, based on extraction by magnetic molecular imprinted microparticles (MagMIP) and subsequent GC-MS analysis.

In the latest years, when developing analytical methods, more and more attention has been posed on innovative extractions which are fast and easy, with a particular interest in environmentally friendly approaches. Among them, magnetic solid phase extraction (MSPE) is a promising technique, because of its ease of application and low consumption of organic solvents. MSPE is based on the use of magnetic nanoparticles or microparticles which are composed of a magnetic core, covered with an adsorbent phase which is able to bind the analytes of interest [120]. The particles are generally

dispersed in the sample and then collected with an external magnetic field; after this step, back extraction is performed prior to analysis. Several adsorbents are used as coatings of magnetic particles, such as silica, polymers and functionalized materials [120,121]. Selectivity is an important aspect to consider when developing this kind of materials, especially if they have to be applied to complex matrices pre-treatment. Great potential in selective or even specific binding properties has been shown by polymers obtained with the molecular imprinting technique [122]. Molecular Imprinted Polymers (MIPs) are tailor-made materials, prepared adding a template molecule to the polymer reaction mixture; after polymerization, the template is washed away, leaving free ‘‘cavities’’ which are chemically and geometrically complementary to the target molecule. The presence of such cavities should favour the selective binding of the template molecule and possibly of molecules belonging to the same class. The combination of MIP and MSPE has been successfully applied for the extraction of specific molecules from environmental and food matrices, bringing the advantages of selectivity and efficiency [123,124]. In the framework of environmental studies, the described approaches are applied to the determination of both emerging and classical pollutants.

Polycyclic aromatic hydrocarbons (PAHs) are a class of contaminants which continue to raise the interest of the scientific community, because of their toxicity and ubiquitous presence in the different environmental compartments [125,126]. PAHs are composed of at least two fused benzenic rings and can be divided in low molecular weight compounds (number of rings minor or equal to four) and high molecular weight compounds (number of rings greater than four). The higher the molecular weight the lower is the vapour pressure and the higher the lipophilicity of PAHs. In general, the lipophilic characteristic causes the accumulation of PAHs in particulate, sediments and organisms; nevertheless, they can be detected in the water compartment at low concentrations. Due to their toxicological profile and suspected carcinogenicity, 16 of them have been classified as priority pollutants by the United States Environmental Protection Agency (US EPA) [127,128]. Although official methods for PAHs determination are still based on liquid-liquid extraction or solid phase extraction [129,130], several innovative techniques, including magnetic approaches, have been proposed in the latest years.

Functionalized silica covered particles [131], polymeric nanoparticles [132] and resin microparticles [133] were applied to PAHs extraction from water samples, as well as other environmental matrices such as soil leachate [134]. Some examples of MIPs application for PAHs extraction can be found as well; usually, only selected PAHs are determined with this approach [135], while the determination of all 16 priority PAHs is less common. In fact, the wide molecular mass range of this compounds, as well as the lack of particular functional groups, make it difficult to find the optimal template molecule [136]. Song et al [137] proposed a MIP synthetized with all 16 PAHs as templates, but this approach is uneconomic and necessarily requires home-made polymers. On the other hand,

commercial magnetic MIPs (magMIPs) microparticles, based on single template imprinting, can be used, but obtaining quantitative recoveries of all 16 PAHs is not straightforward, especially if the classical one variable at a time (OVAT) approach is followed [138].

This chapter deals with the multivariate optimization of the magMIP PAHs extraction. Part of the study aimed to verify the actual advantage of preferring a MIP sorbent to the corresponding non-imprinted material. For the purpose, commercial molecular imprinted microparticles, in which pyrene was used as template molecule, were compared to non-imprinted microparticles (NIP) composed of the same bulk polymer. The Plackett-Burman design was selected to explore the effect of all the variables involved in the process with a low number of experiments. Subsequent tests on the significant variable affecting extraction efficiency were performed to reach optimal recoveries for all PAHs. Finally, the developed magMIP-GC-MS method was applied to some real samples from the Ligurian coast (Italy).

The results presented in this chapter are object of a paper published in an international ISI journal (see Appendix: Publication 4).

8.2 Materials and methods

Chemicals and reagents

A standard solution of PAHs (100 µg mL-1 each in cyclohexane) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). This solution contains the following sixteen PAHs: naphthalene (NA), acenaphthylene (ACL), acenaphthene (AC), fluorene (FL), phenanthrene (PH), anthracene (ANT), fluoranthene (FLT), pyrene (PY), benzo[a]anthracene (BaA), chrysene (CHR), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaPY), indeno[1,2,3-c,d]pyrene (IcdPY), dibenzo[a,h]anthracene (DahA) and benzo[g,h,i]perylene (BghiPE). Dodecane (analytical standard, ≥99.8%) and sodium chloride (≥99%) were purchased from Sigma Aldrich (St. Louis, MO, USA). Sodium sulfate (99%) and potassium chloride (99.5%) were purchased from Carlo Erba Reagenti (Rodano, MI, Italy). Acetone (chromatographic grade) was from VWR Chemicals (Fontenay-sous-Bois, France). Ultra-pure water was obtained from a Millipore Q-Gard system equipped with a Millipak 0,22 µm filter (Millipore, Watford, Hertfordshire, UK).

Magnetic microparticles

Magnetic microparticles were kindly provided by NanoMyp (Granada, Spain). Two types of particles were used: magnetic Molecular Imprinted Polymer (MagMIP) and magnetic Non-Imprinted Polymer (MagNIP). Both are characterized by an average diameter of 3 µm, a magnetite core (γ-Fe_3O_4) and

saturation magnetization of 1.52 emu g-1. The polymeric coating of the microparticles is based on a cross-linked vinylic polymer; in the case of MagMIP the polymer was synthesized with the addition of a template molecule (pyrene), to obtain the molecular imprinting.

Instrumentation and GC-MS analysis

The analyses were performed using a Trace GC Ultra gas chromatograph coupled to an ITQ 1100 ion trap mass spectrometer, both from Thermo Scientific (Rodano, MI, Italy), equipped with an AI-AS 1310 autosampler. The column used was a Thermo Scientific Trace Gold-SQC 30m x 0.25mm ID x 0.25µm (film thickness), with a composition of 95% methyl polysiloxane and 5% phenyl polysiloxane. The following method was used for analysis of PAH: carrier gas was helium at constant flow rate of 1.2 mL min^{-1} and the chromatographic separation was carried out by a 37 minutes run with the following oven program: initial temperature of 60°C (held for 2 minutes), followed by a first ramp to 280°C at 10°C min^{-1} (held for 5 minutes), and a second ramp to 310°C at 10°C min^{-1}, finally held for 10 minutes. The transfer line and ion source temperatures were both set at 250°C. Positive electron ionization mode (EI+) was used with an electron energy of 70 eV. Single ion monitoring (SIM) mode was used to enhance specificity and sensitivity. PAHs are characterized by high stability and do not undergo fragmentation; therefore, the m/z values chosen for the SIM detection were those of the molecular ion; Tables 39 and 40 show the selected m/z for the 16 PAHs, along with their retention times. Benzo[b]fluoranthene and benzo[k]fluoranthene were always quantified together because complete chromatographic separation was not possible with the used capillary column. Xcalibur software was used for data acquisition and processing.

Table 39: Selected ions for SIM detection and retention times of the PAHs under study (molecular weight 128-228).

Compound	NA	ACL	AC	FL	PH	ANT	FLT	PY	BaA
Selected ion (SIM) (m/z)	128	152	154	166	178	178	202	202	228
Retention time (min)	8.23	11.86	12.03	13.49	15.71	15.81	18.52	19.02	21.89

Table 40: Selected ions for SIM detection and retention times of the PAHs under study (molecular weight 252-278).

Compound	CHR	BbF*	BkF*	BaPY	IcdPY	DahA	bBghiPE
Selected ion (SIM) (m/z)	228	252	252	252	276	278	276
Retention time (min)	21.98	24.31	24.31	25.05	28.49	28.91	29.42

* Benzo[b]fluoranthene and benzo[k]fluoranthene were quantified together throughout the work because of partial coelution.

Experimental design

The extraction procedure was based on the addition of the magnetic microparticles to a volume of water, agitation, collection of the particles and back extraction in acetone. Nine variables could potentially affect recovery: type of polymer (molecular imprinted polymer, MIP or non-imprinted polymer, NIP), amount of microparticles/sample volume ratio ($m_{\mu p}/V_{H2O}$), number of extractions (same volume of sea water extracted once or twice with the same quantity of microparticles), extraction time, extraction mode (type of agitation), acetone volume used for back extraction (BE), number of the BEs, BE time and BE mode. Two levels were selected for each variable (the lower coded as -1 and the upper coded as +1) and are presented in Table 41.

Table 41: factors investigated in the experimental design and selected levels.

Factor	-1 level	+1 level
Polymer type	NIP[a]	MIP[b]
$m_{\mu p}/V_{H2O}$ (mg/mL)[c]	0.5	2
Number of Extractions	1	2
Extraction time (min)	15	60
Extraction mode	Soft (gentle rotatory agitation)	Hard (ultrasound extraction)
Back extraction (BE) volume (mL)	0.5	1
Number of BEs	1	3
BE time (min)	1	10
BE mode	Soft (manual agitation)	Hard (ultrasound extraction)

[a] Non-Imprinted Polymer; [b] Molecular Imprinted Polymer; [c] microparticles amount was maintained constant (5 mg), therefore level -1 and +1 for $m_{\mu p}/V_{H2O}$ variable corresponds to 10 mL and 2.5 mL of water sample, respectively.

A Plackett-Burman design was chosen to identify the significant factors out of the nine involved; a total of 12 experiments were performed to study the main effect of the factors. Since only 10 experiments are necessary to determine the coefficients of the factors and the constant term of the model, we had 2 degrees of freedom, associated to two so called “dummy factors”. The experiments were performed in random order to avoid systematic errors due to time effect. The same sea water sample was used in all experiments and consisted of a blank matrix (synthetic sea water) spiked with a known amount of PAHs. The procedure was the following: addition of 10 or 2.5 mL of the spiked sea water to 5 mg of magnetic microparticles (MIP or NIP); agitation in an ultrasonic bath or by

rotatory shaker for 15 or 60 min; recovery of the microparticles with an external ring magnet; drying under N_2 flow to eliminate residual water; 1 or 3 back extractions with 0.5 or 1 mL of acetone (back extraction performed for 1 or 10 minutes in an ultrasonic bath or by manual agitation, both preceded by 1 minute vortex agitation); in the case of two extractions, the sea water already extracted was added to the microparticles again and the whole procedure was repeated. Because of the different values of the variables involved in the extractions, final volumes of the extracts obtained from the 12 experiments were different. In order to maintain the same pre-concentration factor of 10, the final extracts were dried under N_2 and reconstituted with different volumes of acetone (1 mL or 250 µL when the initial volume of water was 10 mL or 2.5 mL, respectively). Dodecane was selected as internal standard (IS) and added to the extracts at concentration of 300 µg L^{-1} to account for possible instrumental fluctuations among different analyses. The extracts were analysed by GC-MS and the areas of single PAH's peaks, normalized to the IS area, were used to perform a principal component analysis (PCA), whose scores on the first principal component served as the response to be modelled. The open-source software "R", (R core team, 2014) was used for computation of the models and to perform PCA.

Extraction procedure

After the experimental design, only one variable was further optimized, namely the amount of microparticles/sample volume ratio. The optimal value of this factor was 0.25 and 1, for compounds ranging from m/z 128 to 228 ("light" PAHs) and from m/z 252 to 278 ("heavy" PAHs), respectively. Hence, the final proposed extraction procedure involved the use of 5 mg of MagMIP microparticles for the extraction of "light" PAHs from 20 mL of seawater, while 20 mg of microparticles for the extraction of "heavy" PAHs from 20 mL of seawater. In both cases, a small volume of acetone (80 µL) was added before sample introduction, to allow a complete dispersion of the MagMIP in water. Samples were gently shaken during 15 minutes through a rotary agitator to foster PAHs adsorption onto the microparticles. MagMIP were easily collected with the aid of an external ring magnet, in which the vial containing the sample was inserted (Fig. 39). The magnetic field makes the microparticles stick to the walls of the vial, allowing an easy discarding of the water with no particles loss.

Figure 39: dispersion of the microparticles in water and collection with the external magnet.

Back extraction of PAHs was performed with 0.5 mL of acetone and vortex agitation for 1 minute. This step was repeated twice, and the supernatants mixed together (1 mL of total extract). Finally, the solution was filtered through a 0.22 µm PTFE (polytetrafluoroethylene) filter and the internal standard (dodecane) was added at a concentration of 300 µg L^{-1} before GC-MS analysis.

Standards and calibration

Working standard solutions were prepared in acetone starting from a concentrated standard mix at 100 µg mL^{-1}. Solutions at 2, 5, 25, 50, 100 and 200 µg L^{-1} were used to build the calibration curves and establish the linearity ranges. Limit of detection (LOD) and limit of quantitation (LOQ) of the analytes were calculated considering a signal to noise ratio of 3 and 10 respectively. LOD values were verified by injecting standards at the LOD concentration in triplicate. Instrumental precision was evaluated as intra-day repeatability (3 replicate injections for all levels of concentration) and inter-days repeatability (3 days, 3 replicates per day for two levels of concentration). Recovery (accuracy) were evaluated for the two optimized protocols (one for PAHs with mass up to 228, and one for PAHs with mass from 252 to 278) by spiking a blank sea water sample with known amount of PAHs (5 µg L^{-1}) and comparing the expected concentration values with the measured ones. Precision of the whole analytical method (procedural precision) was assessed by performing 6 replicate extractions of the same spiked sample (PAHs concentration at 2.5 µg L^{-1}).

Synthetic sea water and real samples

The first tests to determine recovery of the extraction procedure were performed using synthetic sea water as blank matrix. A simple formula, which includes the main marine salts, was used for the preparation of this matrix: 22.64 g of NaCl, 0.78 g of KCl and 4.15 g of (Na_2SO_4) were added to 1 liter of milli-Q water. Real sea water samples were collected in the port area of Genoa (Liguria, Italy),

using glass bottles, and kept at 4°C in the dark. Extraction and GC-MS analysis were performed within 2 days from collection, to avoid any degradation.

8.3 Results and discussion

The simultaneous extraction of 16 PAHs from sea water required a careful study, in order to obtain acceptable recoveries for all compounds. A simple procedure for PAHs extraction was described by the manufacturer which provided the magnetic microparticles; nevertheless, the first tests showed poor recovery for some PAHs and suggested that the procedure could be improved by a systematic study of the variables involved. First of all, extraction with magMIP and magNIP was compared, then an experimental design allowed to estimate the effect of various factors on the PAHs recovery. Only one factor was found to be significant and it was studied in an appropriate range, to identify the best value for maximum recovery of the 16 analytes.

MIP/NIP comparison

MagMIP and magNIP microparticles differ for the technique used to synthesize the polymeric coating of the magnetic core; for magMIP preparation a template molecule is added to the polymer synthesis mix to obtain the molecular imprinting, while for magNIP no template is added, resulting in the same polymer bulk, but with no "cavities". Theoretically, in MIPs the absorption of analytes with a structure similar to the template should take place at the cavities, with a very specific behavior. However, the polymeric matrix itself could act as a sorbent phase and non-specific weak interaction could lead to adsorption of the analytes onto the polymer. We compared extraction efficiency and repeatability of the same extraction protocol, by changing only the type of microparticles (MIP or NIP), to verify how non-specific interactions influence adsorption and consequently extraction of PAHs. The protocol proposed by the magMIP manufacturer consisted of the following steps: dispersion of 10 mg of magMIP or magNIP microparticles in a 100 μL of acetone and addition of 10 mL of spiked synthetic sea water extraction for 45 minutes in an ultrasonic bath; recovery of the microparticles and discard of the water; back extraction in 1 mL of acetone. Three replicates were performed both for MIP and NIP extraction and recovery was evaluated by comparing measured and theoretical concentration in the acetone extracts. The percent recoveries obtained by the two methods are shown in Fig.40.

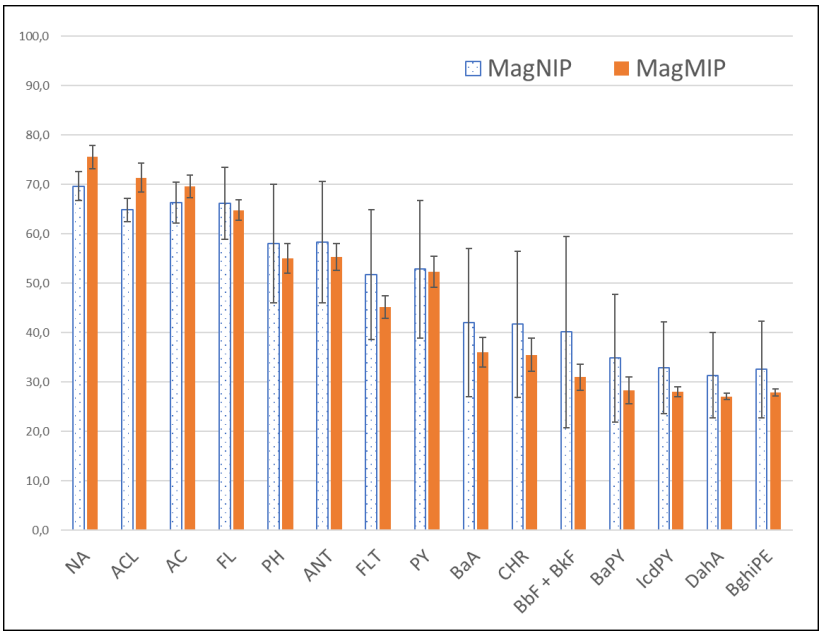

Figure 40: Comparison between magMIP and magNIP extraction: average percent recoveries of three extractions.

For PAHs with molecular weight from 128 to 202, recovery values were in the range of 55-70% and 56-76% for magNIP and magMIP, respectively; for PAHs with molecular weight from 228 to 278, recoveries were in the range of 32-45% and 28-36% for magNIP and magMIP, respectively. A t-test was performed to compare the results of the two experiments: no significant difference was found in the recovery of the PAHs. This means that all compounds interacted with the bulk material, giving similar recoveries both with MIP and NIP extraction. As for repeatability of the two methods, a considerable difference was observed; in fact, while replicates of the MIP extractions on the same sample led to relative standard deviation (RSD) of 0.6 - 4 % for all analytes, NIP extraction replicates gave RSD of 3-7% for naphthalene, acenaphthylene, acenaphthene and fluorene and RSD of 9-22% for the other PAHs. These results showed that, although recoveries were not significantly different between the two methods, the non-specific adsorption of the analytes onto the NIP surface caused a higher variability in extraction efficiency; for this reason, the use of MIP should be preferred.

Results of the experimental design

The extraction protocol followed in the preliminary tests led to recoveries of 55-75% for PAHs with molecular weight from 128 to 202 and of 30-40% for PAHs with molecular weight from 228 to 278. In order to improve the extraction efficiency of the methodology, the multivariate approach of experimental design was chosen; in particular, a Plackett-Burman design was used to examine all the 9 factors possibly influencing the response. The levels of each variable (Table 41), were selected as to explore a domain wide enough to highlight detectable effects on the response. Both qualitative and quantitative variables were investigated: qualitative variables were the type of

polymer (NIP or MIP), the extraction mode and the BE mode; the discrete quantitative variables were the number of extractions (1 or 2) and the number of back extraction (1 or 3); the continuous quantitative variables were the ratio among microparticles amount and volume of water, the extraction time, the BE time and the volume of acetone used for BE. Even though MIP and NIP were already compared in previous tests (section "MIP/NIP comparison"), we decided to include the variable "type of polymer" in the design to verify the results already obtained. The experimental domain selected for the variables was centred at the values already tested in the preliminary studies, choosing a range which was reasonable from a practical point of view. Table 42 shows the experimental matrix of the design, with non-coded levels (i.e. the real values of each variable under study), thus indicating the procedure followed for each experiment.

Table 42: experimental matrix for the Plackett-Burman design.

Factors →	Polymer type	$m_{\mu p}/V_{H2O}$ (mg/mL)	Number of Extractions	Extraction time (min)	Extraction mode	BE volume (mL)	Number of BEs	BE time (min)	BE mode	Dummy factors	
EXP	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}
1	MIP	2	1	60	hard	1	1	1	soft	+1	-1
2	NIP	2	2	15	hard	1	3	1	soft	-1	+1
3	MIP	0.5	2	60	soft	1	3	10	soft	-1	-1
4	NIP	2	1	60	hard	0.5	3	10	hard	-1	-1
5	NIP	0.5	2	15	hard	1	1	10	hard	+1	-1
6	NIP	0.5	1	60	soft	1	3	1	hard	+1	+1
7	MIP	0.5	1	15	hard	0.5	3	10	soft	+1	+1
8	MIP	2	1	15	soft	1	1	10	hard	-1	+1
9	MIP	2	2	15	soft	0.5	3	1	hard	+1	-1
10	NIP	2	2	60	soft	0.5	1	10	soft	+1	+1
11	MIP	0.5	2	60	hard	0.5	1	1	hard	-1	+1
12	NIP	0.5	1	15	soft	0.5	1	1	soft	-1	-1

The Plackett-Burman design evaluates only main effects, therefore the model of the response as a function of the variables simply consists of linear terms, with no interactions.

Theoretically, since 15 responses were considered (PAHs chromatographic peak areas), 15 models should be computed, but if some of the responses were correlated, they could be grouped, computing a smaller number of models. Actually, the responses to evaluate were 12, since the evaporation step involved in the experiments caused the loss of naphthalene, acenaphthylene and acenaphthene. To reduce the number of responses to be considered, a PCA was performed on the data obtained from the Plackett-Burman experiments. It allowed to find a correlation among the peak areas of the PAHs and, therefore, an easier way to represent the results of the experiments. The data matrix subjected to PCA consisted of 12 rows (experiments of the Plackett-Burman design) and 12 columns (variables,

namely the areas of the 12 PAHs), and a constraint of 5 principal components was set. It was found that the first two PCs explained the 97.2% of the total variance. This result allowed to express the data eliminating a percentage of variability related to noise and highlighted the strong correlation among the 12 PAHs areas. By looking at the loading plot in Fig. 41, we deduced that the original variables were all positively related to the first PC (PC1, explaining the 81.8% of variability), and correlated among themselves. Moreover, there was a clear separation of two groups on the second PC (PC2), the first corresponding to PAHs with mass between 128 and 202, and the second to PAHs with mass between 228 and 278.

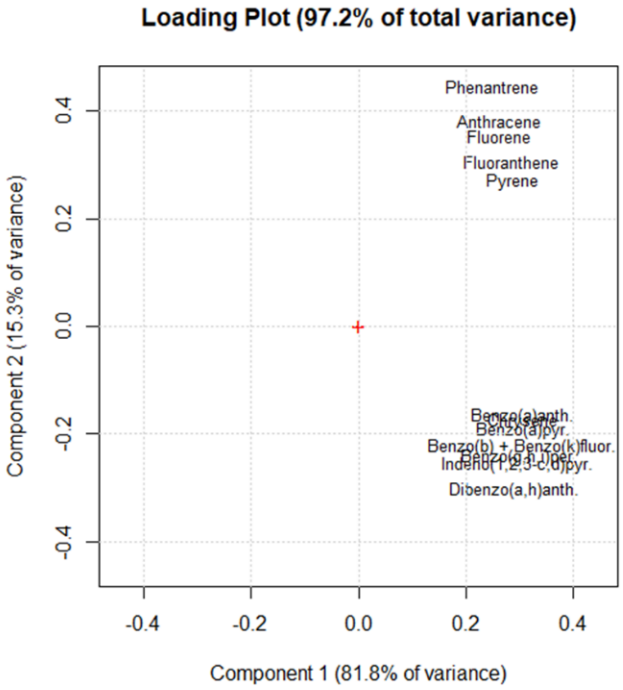

Figure 41: Loading plot of the PCA performed on the results of the Plackett-Burman design. The variables in the loading plot are the PAHs peak areas.

This division was already suggested by the fact that heavier PAHs had all smaller areas than the lighter ones. Since a very high percentage of variability was explained by PC1, the scores of the experiments on this PC, were considered as a very good approximation of the original data. Therefore, the scores on PC1 of the 12 experiments were selected as a unique response to be modelled, instead of the single PAHs areas. The model was computed and since 12 experiments were performed to obtain 10 coefficients, two additional coefficients, related to dummy factors, were estimated. Each coefficient value was compared to the coefficients of the dummy factors to evaluate significance; the values of all the coefficients are presented in Table 43.

Table 43: Coefficients of the linear model obtained from the results of the Plackett-Burman design (in bold the unique significant factor).

Factor	Coefficient of the linear model
Polymer type[a]	1.15
$m_{\mu p}/V_{H2O}$[b]	**1.53**
Number of Extractions	0.98
Extraction time	0.29
Extraction mode	0.32
BE[c] volume	0.65
Number of BEs	1.01
BE time	-0.86
BE mode	0.50
Dummy factor 1	*-1.30*
Dummy factor 2	*0.32*

Only the variable "amount of microparticles/sample volume ratio" ($m_{\mu p}/V_{H2O}$) out of the nine studied had a coefficient greater than both dummy factors. This indicated that a change in the other variables produced an effect smaller than experimental variability. Hence, in the following experiments, the most convenient value (from a practical point of view) of the non-significant variables was selected, while the effect of different $m_{\mu p}/V_{H2O}$ ratios was investigated.

It is noteworthy that the experimental design confirmed the results already obtained with the comparison of MIP and NIP extraction. The non-significance of the qualitative factor "type of polymer" (MIP or NIP) indicates that recovery was not statistically different if MIP or NIP was used for PAHs extraction. Nonetheless, as already stated in the previous paragraph, NIP showed poor repeatability; for this reason, only MIP were used in the following experiments.

Effect of the $m_{\mu p}/V_{H2O}$ ratio

To study the effect of the ratio among the amount of microparticles and the volume of the sea water sample ($m_{\mu p}/V_{H2O}$) on PAHs recovery, all the other variables were kept constant while the level of this variable was changed. After performing a screening design, the coefficients of the model can give a suggestion on the effect of the variables: the sign of the coefficient indicates the direction to follow to obtain a better response. In the case of the $m_{\mu p}/V_{H2O}$ variable, the positive sign of the coefficient suggested that an increase in the amount of microparticles added to a certain volume of water would produce an improvement in extraction efficiency. To verify this hypothesis, a quite large range of the variable was investigated. Five tests in duplicate were performed with values of $m_{\mu p}/V_{H2O}$ of 3, 2, 1,

0.5 and 0.25 mg mL^{-1}. A blank sample of real sea water was spiked with PAHs at a concentration of 5 µg L^{-1} and extraction was performed on 20 mL of water, agitating for 15 minutes, recovering the microparticles with a ring magnet and performing two consecutive BEs with 0.5 mL of acetone each. The percent recoveries of the 5 tests are represented in the graph in Fig. 42.

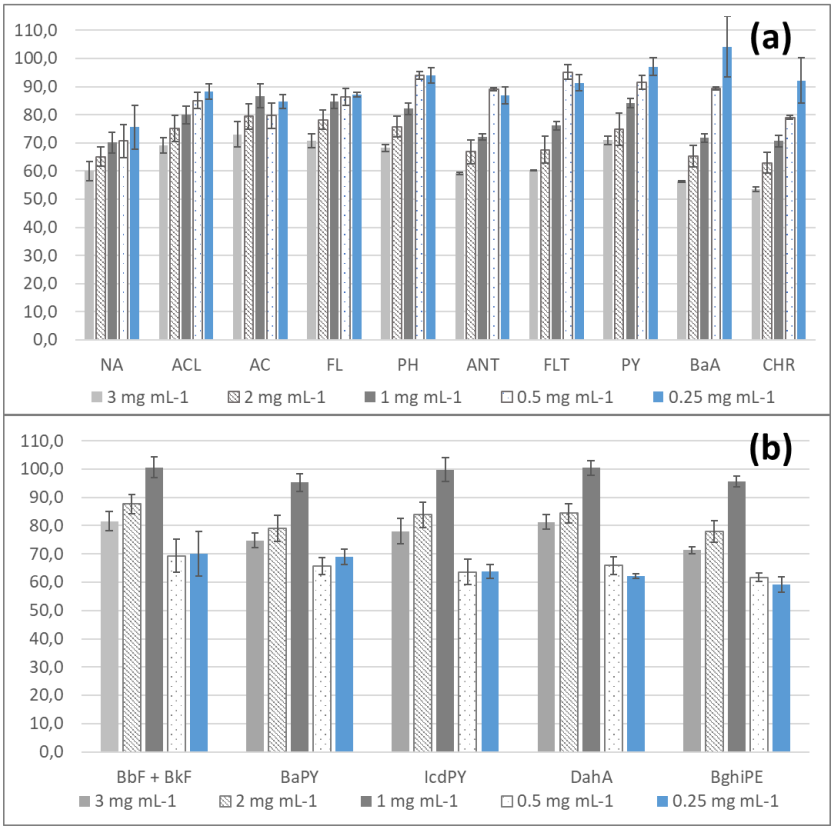

Figure 42: Percent recoveries of PAHs with mass from 128 to 228 (a) and from 252 to 278 (b) obtained by the tests performed at $m_{\mu p}/V_{H2O}$ ratios of 3, 2, 1, 0.5 and 0.25 mg L^{-1}.

Interestingly, for PAHs with mass between 128 and 228 the results showed an inversion in the effect of the variable with respect to the results of the experimental design; higher recoveries were observed by decreasing the amount of microparticles used for a fixed volume of water and the best recovery values were obtained by using a $m_{\mu p}/V_{H2O}$ ratio of 0.25, with values ranging from 76 to 104%. This apparent inversion in the effect of the variable, could be due to the volume of acetone used for the back extraction. In fact, by changing the $m_{\mu p}/V_{H2O}$ ratio but keeping the BE volume of acetone at 1 mL, the ratio among amount of microparticles and volume of acetone changed as well. We supposed that an interaction among the variable $m_{\mu p}/V_{H2O}$ and the volume of acetone used in the BE was present. Since the Plackett-Burman design allows to estimate main effects but not interaction, we were not able to detect this effect, which could actually be greater than the one given by the variable $m_{\mu p}/V_{H2O}$; this could explain the better recoveries attained when smaller ratio of magMIP amount and acetone volume was used. On the other hand, this behaviour was only partially observed for PAHs with mass

between 252 and 278. For these compounds maximum recoveries were reached by using a $m_{\mu p}/V_{H_2O}$ ratio of 1, with values ranging from 95 to 101%. When $m_{\mu p}/V_{H_2O}$ ratio smaller than 1 was used, recovery began to decrease. This phenomenon might be explained by the different interaction of the heavier PAHs with the MIP surface. Because of the different effect of the $m_{\mu p}/V_{H_2O}$ ratio for low and high molecular weight PAHs, it was not possible to optimize a unique procedure which led to maximum recoveries of all the compounds. For the extraction of PAHs with mass from 128 to 228, 5 mg of magMIP are recommended for 20 mL of sea water sample; for the extraction of PAHs with mass from 252 to 278, 20 mg of magMIP should be used for 20 mL of sea water sample. The remaining part of the optimal procedure was common both for the extraction of light and heavy PAHs and was presented in the materials and methods section.

Analytical performances

The main figures of merit of the analytical method were evaluated: linearity range, LODs and LOQs, inter-day and intra-day precision. The recovery and procedural precision of the two optimal procedures (for light and heavy PAHs) were obtained as well. Table 44 reports these parameters for the 16 PAHs, with LODs and LOQs expressed considering the pre-concentration factors.

Table 44: Analytical performances of the proposed magMIP-GC-MS method.

Compound	R^2	Linearity (µg L^{-1})	LOD (µg L^{-1})	LOQ (µg L^{-1})	Intra-day Precision (RSD%)	Inter-day Precision (RSD%)	Recovery (%)	Procedural precision (RSD%)
naphtalene	0.9996	5-200	0.075	0.250	3.0	6.7	75.6	10.9
acenaphthylene	0.9999	10-200	0.150	0.500	3.3	7.0	88.2	5.0
acenaphthene	0.9998	5-200	0.075	0.250	3.3	8.8	84.8	6.9
fluorene	0.9999	5-200	0.075	0.250	3.5	8.2	87.2	4.3
phenanthrene	0.9998	5-200	0.075	0.250	2.7	8.7	94.0	4.4
anthracene	0.9998	5-200	0.075	0.250	3.3	7.1	86.9	4.0
fluoranthene	0.9998	2-200	0.030	0.100	3.4	6.8	91.4	3.9
pyrene	0.9996	2-200	0.030	0.100	3.5	5.7	97.1	3.7
benzo[a]anthracene	0.9996	5-200	0.075	0.250	4.7	8.7	104.1	8.0
chrysene	0.9987	5-200	0.030	0.100	4.4	8.4	92.2	8.0
benzo[b]fluoranthene + benzo[k]fluoranthene	0.9984	50-400	0.750	2.50	6.0	13.9	100.6	10.3
benzo[a]pyrene	0.9971	25-200	0.375	1.25	6.0	14.9	95.2	9.4
indeno[1,2,3-c,d]pyrene	0.9942	25-200	0.375	1.25	6.0	11.6	99.8	10.6
dibenzo[a,h]anthracene	0.9906	25-200	0.375	1.25	5.5	11.7	100.5	11.9
benzo[g,h,i]perylene	0.9950	25-200	0.375	1.25	7.8	9.5	95.6	12.2

The linearity of the calibration curves was verified in the range of 2-200 µg L^{-1} for fluoranthene and pyrene, 5-200 µg L^{-1} for naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, benzo[a]anthracene and chrysene, 10-200 µg L^{-1} for acenaphtylene; smaller ranges of linearity were observed for PAHs with mass between 252 and 278 (25-200 µg L^{-1}). Good determination coefficient (R^2) were obtained for all calibration curves, with the majority of the values greater than 0.999, and all above 0.99. Very good sensitivity was reached thanks to the enrichment factor of 20 as well as the use of the SIM method, which limited the background signal of the chromatogram, enhancing the signal to noise ratio (S/N).

LODs and LOQs were in the range of 0.03-0.75 µg L^{-1} and 0.1-2.5 µg L^{-1} respectively; these values are lower than the ones of the official EPA methods, except for the high molecular PAHs for which the fluorimetric detection allows a better sensitivity. Moreover, they are comparable to the ones obtained with other GC-MS methods present in the literature, which are in the range of some ng L^{-1} to µg L^{-1}[133,137,139–142], commonly associated with high preconcentration factors.

Intra-day (3 replicates) precision was assessed at all concentration levels of the calibration curves, while inter-day precision (3 days, 3 replicates per day) was assessed at two concentration levels (25 and 100 µg L^{-1}); average relative standard deviations (RSD%), were in the range of 2.7-7.8% and 5.7-14.9% respectively. The recovery values of the optimized extraction protocols (one for PAHs with mass up to 228, and one for PAHs with mass from 252 to 278) were all comprised between 75.6% and 104.1%, better or comparable to the values reached by Villar-Navarro et al with the same magMIP particles [138]. As for procedural precision, obtained by 6 replicate quantitation of the same spiked sample, RSD values were all smaller or equal to 12%.

Application to sea water samples

The applicability of the proposed method was tested on three sea water samples, collected in the port of Genova. The samples consisted of surface water taken from potentially impacted areas, i.e. near to a petrol station for boat refueling (sample 1), in the estuarine of a creek (sample 2) and inside the port area dedicated to boarding and transfer of petrol products. We did not expect detectable concentrations of heavy PAHs, since their more lipophilic nature causes accumulation in sediments and organisms[143]. On the other hand, low molecular weight PAHs tends to preferentially distribute in the dissolved phase [144,145]. Moreover, during a previous study carried out by our group on seawater collected in the same area (unpublished data), we did not detect any PAH with molecular weight higher than 202. Therefore, we decided to apply the protocol more suitable for PAHs with mass from 128 to 228. The results of the analyses are shown in Table 45.

Table 45: Results of the analyses performed applying the optimized method on sea water samples from the port area of Genoa, Italy.

Compound	Sample 1 (µg L^{-1})	Sample 2 (µg L^{-1})	Sample 3 (µg L^{-1})
naphtalene	0.97 ± 0.18	0.40 ± 0.18	< LOQ
phenantrene	0.37 ± 0.11	0.29 ± 0.11	0.30 ± 0.11
pyrene	0.51 ± 0.16	0.30 ± 0.16	0.46 ± 0.16

Only naphthalene, phenantrene and pyrene were above LOQs in the analyzed samples. The three sampling areas should be characterized by different impact from punctual sources. Nonetheless, the concentration of the detected PAHs in the samples were comparable. This might suggest that discharges from punctual sources only partially contribute to the PAHs contamination of sea water. In fact, the detected compounds are generally among the most important PAHs found in the air compartment and in precipitation, and their presence in the atmosphere contributes to the marine pollution via dry and wet deposition [146]. Therefore, the detection of the three PAHs in the analyzed samples could also be correlated to this phenomenon.

8.4 Conclusions

In this research, a multivariate optimization strategy was followed to attain maximum recoveries of 16 PAHs, when performing an extraction by magnetic-MIP microparticles. The method was studied for the application to sea water samples, where low concentrations of PAHs are expected. The results obtained from both preliminary tests and the experimental design showed that the use of MIPs rather than non-imprinted materials did not bring significant improvement in extraction efficiency for the majority of PAHs. Nevertheless, MIPs proved to be more reliable in terms of repeatability. Attaining quantitative recoveries for all the 16 PAHs was a difficult task, due to the different shape and mass of the molecules, which interact differently with the MIP surface. The final protocol resulted in two different amounts of microparticles, optimal for a 20 mL sea water sample, depending on the PAHs to be determined (low or high molecular weight). Good figures of merit were achieved for the proposed method, which proved to be reliable, fast and environmentally-friendly, thanks to the low volume of acetone (1 mL) involved in the extraction procedure. The application to some sea water samples showed that naphthalene, phenantrene and pyrene were present at trace levels in waters belonging to an Italian port area.

This study, not directly related to the main body of the thesis, demonstrated once again the potential of multivariate optimization when a high number of variables are involved in a procedure. It also showed the importance of other multivariate tools (PCA), useful when we need to interpret data depending on several variables, but probably correlated among each other.

Chapter 9

General conclusions

During this PhD work, a range of analytical problems were faced. Chromatographic methods coupled to mass spectrometry were developed and used for several applications. New or improved strategies and methods were proposed with respect to the existing literature, bringing valuable extension of the current knowledge. The main focus was on phytoestrogens determination in soy food matrices; in addition, a work on an environmental problem, namely the presence of PAH in sea water, was performed. The two topics shared the common strategy of studying the pre-treatment steps by experimental design to obtain a comprehensive and rapid optimization, as well as the investigation on unconventional extraction techniques; these have been combined with chromatography-mass spectrometry, to develop innovative, sensitive and specific analytical methods.

The first research line regarded the development and comparison of a GC-MS and a LC-MS method for the determination of daidzein, genistein, formononetin, biochanin A and coumestrol in soy-milk. As for the GC-MS method, a wide investigation was carried out to find the best instrumental conditions for the analysis of phytoestrogens after derivatization. Tandem MS was exploited to reach maximum sensitivity and specificity, and the influence of other parameters (such as ion source

temperature and injection mode) on the analytical responses was systematically evaluated. Once this aspect was optimized, a simple experimental design was planned to study the effect of the variables influencing the derivatization step preceding the GC analysis. The multivariate approach allowed to discover that a much faster and simpler procedure could be used for phytoestrogens derivatization, thus introducing a significant novelty and improvement in this kind of determination. In fact, the works present in literature described long and difficult derivatization processes which made this analysis impractical. Unfortunately, some problems were encountered for genistein and biochanin A determination, due to the instability of the derivatives formed.

The development of a LC-MS method allowed to overcome this problem. A sensitive determination based on the multiple reaction monitoring mode permitted to attain precision and accuracy for all analytes. A comparison among the methods figures of merits highlighted the pros and cons of the two techniques. Some soy-milks were analysed by both methods, and results were critically compared. At first, the analytes separation was achieved by using a classical reverse phase chromatographic column (C18). Although the method was satisfactory, we explored the possibility of improving it by testing a different column, theoretically more suitable for the phytoestrogens under study, based on their physico-chemical characteristics. Indeed, by using a PFP column, it was possible to obtain a shorter chromatographic run and a simpler mobile phase gradient, as well as more sharp and symmetric peaks.

After enhancing the method performances, the focus was moved to a particular soy-based matrix, whose diffusion in the Italian market has recently grown, i.e. soy-based burgers. To the best of our knowledge, no method was found in the literature which put the emphasis on this kind of matrix. Its complexity made it necessary a proper study to find the best sample pre-treatment to reach good recoveries and matrix effect. For this aim, the work was organized in two stages: firstly, three extractions methodologies were compared (UAE, SPE and QuEChERS), after applying some modified literature methods (used for other samples); secondly, the most innovative technique among the three, namely QuEChERS, was carefully optimized in terms of recovery and matrix effect of the five analytes. The multivariate approach was exploited by performing two sequential experimental designs, which permitted a reliable, rational and fast optimization of the pre-treatment of soy-burgers before LC-MS/MS analysis.

The optimal conditions found were applied for the quantitation of phytoestrogens in several products belonging to the Italian market, treating the soy-burgers both raw and after cooking. A chemometrical tool (PCA) was used to look for patterns into the group of studied samples, and no difference was highlighted between raw and cooked burgers, demonstrating phytoestrogens stability.

Finally, the environmental study on PAH determination in sea water by GC-MS, which was not correlated to the main part of the thesis, demonstrated once again the importance of multivariate optimization when innovative methods depending on several variables must be developed. Thanks to a simple and fast screening design it was possible to identify the only important variable affecting extraction efficiency of the mag-MIP extraction, among nine theoretically influencing factors. A fast and green optimized method for PAHs quantitation in sea water was proposed.

This thesis shows the importance of a comprehensive and rational optimization of both analytical instrumental techniques and extraction strategies, to attain consistent results when dealing with trace analysis in food and environmental samples.

Appendix: Publications

1) Emanuele Magi, Marina Di Carro, Cristiana Mirasole, **Barbara Benedetti**.
   Combining passive sampling and tandem mass spectrometry for the determination of pharmaceuticals and other emerging pollutants in drinking water. Microchemical Journal, 136C (2018) pp. 56-60.
   https://doi.org/10.1016/j.microc.2016.10.029

2) **Barbara Benedetti,** Marina Di Carro, Cristiana Mirasole, Emanuele Magi.
   Fast derivatization procedure for the analysis of phytoestrogens in soy milk by gas chromatography tandem mass spectrometry. Microchemical Journal, 137 (2018) pp. 62–70. https://doi:10.1016/j.microc.2017.09.023.

3) **Barbara Benedetti,** Marina Di Carro, Emanuele Magi.
   Phytoestrogens in soy-based meat substitutes: Comparison of different extraction methods for the subsequent analysis by liquid chromatography-tandem mass spectrometry. Journal of Mass Spectrometry 53 (2018) pp. 862-870.
   https://doi:10.1002/jms.4268.

4) **Barbara Benedetti,** Marina Di Carro, Emanuele Magi.
   Multivariate optimization of an extraction procedure based on magnetic molecular imprinted polymer for the determination of polycyclic aromatic hydrocarbons in sea water. Microchemical Journal 145 (2019) pp. 1199-1206.
   https://doi.org/10.1016/j.microc.2018.12.048.

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