NATIONAL AND KAPODISTRIAN UNIVERSITY OF ATHENS

SCHOOL OF SCIENCES

DEPARTMENT OF CHEMISTRY

MASTER OF SCIENCE IN "ANALYTICAL CHEMISTRY AND QUALITY ASSURANCE"

MASTER THESIS

Method Development for the Determination of Water-soluble Vitamins in Cereal by Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS)

NATALIA PAPALOUKA
CHEMIST

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NATALIA PAPALOUKA

REGISTRATION NUMBER: 182212

SUPERVISOR PROFESSOR:

Nikolaos S. Thomaidis, Professor NKUA

THREE-MEMBER CONSULTATIVE COMMITTEE:

Evangelos Gikas, Assistant Professor NKUA

Anastasios Oikonomou**,** Professor NKUA

Nikolaos S. Thomaidis, Professor NKUA

DEFENDING DATE: 04/02/2021

ΕΡΕΥΝΗΤΙΚΗ ΕΡΓΑΣΙΑ ΔΙΠΛΩΜΑΤΟΣ ΕΙΔΙΚΕΥΣΗΣ

Ανάπτυξη μεθόδου για τον προσδιορισμό υδατοδιαλυτών βιταμινών σε δημητριακά με χρήση Υγροχρωματογραφίας συζευγμένη με Φασματομετρία Μάζας (LC-MS/MS)

ΝΑΤΑΛΙΑ ΠΑΠΑΛΟΥΚΑ

Α.Μ.: 182212

ΕΠΙΒΛΕΠΩΝ ΚΑΘΗΓΗΤΗΣ:

Νικόλαος Σ. Θωμαΐδης, Καθηγητής ΕΚΠΑ

ΤΡΙΜΕΛΗΣ ΕΞΕΤΑΣΤΙΚΗ ΕΠΙΤΡΟΠΗ:

Ευάγγελος Γκίκας, Επίκουρος Καθηγητής ΕΚΠΑ

Αναστάσιος Οικονόμου**,** Καθηγητής ΕΚΠΑ

Νικόλαος Σ. Θωμαΐδης, Καθηγητής ΕΚΠΑ

ΗΜΕΡΟΜΗΝΙΑ ΕΞΕΤΑΣΗΣ: 04/02/2021

ABSTRACT

During the last decades, vitamins have been utilized as additives in foods, animal feed and dietary supplements, in order to enhance the consumers' recommended intake, since they cannot synthesize them on their own. Vitamins have a vital role for normal health and growth of living organisms and owing to their solubility, they are classified as water-soluble (B-complex and C) and fat-soluble (A, D, E and K). These compounds are susceptible to degradation by chemical and physical factors. For this reason, the content of vitamins in foodstuff should be checked continuously so as to comply with the labels' values. Thus, there is need for reliable methods for the determination of vitamins in various food matrices such as cereals, dairy products, baby foods etc.

The aim of this study was the development of a rapid, sensitive, selective, cost-effective analytical method for the determination of the water-soluble vitamins (B1, B2, B3, B5, B6, B7, B9 and C) in cereals using Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS).

Initially, an extended review of water-soluble vitamins and the existing methodologies for their determination is presented. The experimental section of the thesis is constituted of two main parts: (1) Development and optimization of an analytical method for the determination of 8 water-soluble vitamins and (2) Method validation.

In particular, different extraction procedures were tested and various parameters such as the weight of sample, the extraction solvent, and the time of extraction were optimized as well as the chromatographic conditions. Finally, validation experiments were conducted to ensure the precision of the method and it can be applied as routine analysis.

SUBJECT AREA: Analytical Chemistry

KEYWORDS: LC-MS/MS, water-soluble vitamins, liquid-liquid extraction, cereal

ΠΕΡΙΛΗΨΗ

Τις τελευταίες δεκαετίες, οι βιταμίνες χρησιμοποιούνται ως πρόσθετα σε τροφές, ζωοτροφές και συμπληρώματα διατροφής, με σκοπό να αυξήσουν την απαιτούμενη πρόσληψη των καταναλωτών, αφού ο οργανισμός τους δεν δύναται να τις συνθέσει μόνος του. Οι βιταμίνες παίζουν ζωτικό ρόλο για την υγεία και τη φυσιολογική ανάπτυξη των ζωντανών οργανισμών και εξαιτίας της διαλυτότητάς τους, ταξινομούνται σε υδατοδιαλυτές (Β-σύμπλεγμα και C) και λιποδιαλυτές (A, D, E και K). Οι ενώσεις αυτές είναι ευαλλοίωτες λόγω χημικών και φυσικών παραγόντων. Γι' αυτό το λόγο, θα πρέπει να ελέγχεται συνεχώς το περιεχόμενο των βιταμινών στα τρόφιμα ώστε να συμμορφώνονται με τις αναγραφόμενες τιμές στις ετικέτες. Συνεπώς, υπάρχει ανάγκη για αξιόπιστες μεθόδους για τον προσδιορισμό των βιταμινών σε διάφορα τρόφιμα, όπως τα δημητριακά , τα γαλακτοκομικά προϊόντα, οι παιδικές τροφές κλπ.

Στόχος της παρούσας μελέτης ήταν η ανάπτυξη μιας ευαίσθητης, επιλεκτικής, οικονομικής αναλυτικής μεθόδου για τον προσδιορισμό των υδατοδιαλυτών βιταμινών (B1, B2, B3, B5, B6, B7, B9 και C) σε δημητριακά με τη χρήση Υγροχρωματογραφίας συζευγμένη με Φασματομετρία Μάζας (LC-MS/MS).

Αρχικά, παρουσιάζεται μια εκτεταμένη ανασκόπηση των βιταμινών και υπαρχουσών μεθοδολογιών που έχουν εφαρμοσθεί για τον προσδιορισμό τους. Το πειραματικό μέρος της διατριβής αποτελείται από δύο κύρια μέρη: (1) Ανάπτυξη και βελτιστοποίηση μιας αναλυτικής μεθόδου για τον προσδιορισμό 9 υδατοδιαλυτών βιταμινών και (2) Επικύρωση της μεθόδου.

Πιο συγκεκριμένα, ποικίλες εκχυλίσεις δοκιμάστηκαν και διάφοροι παράμετροι όπως η μάζα του δείγματος, ο διαλύτης εκχύλισης και ο χρόνος εκχύλισης βελτιστοποιήθηκαν, καθώς και οι χρωματογραφικές συνθήκες. Τέλος, πραγματοποιήθηκαν πειράματα επικύρωσης ώστε να ελεγχθεί η ακρίβεια της μεθόδου και να μπορεί να εφαρμοστεί ως ανάλυση ρουτίνας.

ΘΕΜΑΤΙΚΗ ΠΕΡΙΟΧΗ: Αναλυτική χημεία

ΛΕΞΕΙΣ-ΚΛΕΙΔΙΑ: LC-MS/MS, υδατοδιαλυτές βιταμίνες, εκχύλιση υγρού-υγρού, δημητριακά

Στην οικογένεια μου

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PREFACE

This work was conceived and performed at the Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Greece under the supervision of Professor Nikolaos S. Thomaidis.

This thesis would not have been possible without the help and support of my supervisor, Dr. Nikolaos Thomaidis. I would like to thank him, for his guidance during my master. I am very happy to be part of his research group and grateful for all the opportunities and trust that I received. Also, I especially thank the members of the examination committee, Professor E. Gikas and A. Oikonomou, for their comments and remarks regarding my master thesis.

These past two years were very important in order to develop and improve my theoretical knowledge and practical skills in the field of Analytical Chemistry. I would like to especially thank my supervisor PhD candidate M. Katsa for her help and enormous support. The good advice, encouragement, and immense knowledge of her have been invaluable on both an academic and a personal level and were key motivations throughout my master.

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CHAPTER 1

VITAMINS IN CEREAL

1.1 Introduction

In the last decades, consumer demands in the field of food production has changed considerably [1], [2]. Because of new lifestyles and increasing purchasing power, health-conscious consumers are interested in foods that are appropriate to prepare and maintain a fresh-like quality and contain natural ingredients. Today foods are not intended only to satisfy hunger and provide necessary nutrients for humans, but also to prevent nutrition-related diseases and improve physical and mental well-being of the consumers [3]–[5]. So, the food industry has developed techniques for producing minimally processed food products (MPF) of better quality and with an extended shelf life sufficient in order to make their distribution feasible [6]. One important nutrient in these products is vitamins. Vitamins are organic compounds that are classified into two main groups: fat-soluble and water-soluble. They are essential in small amounts to sustain life and good health and especially during pregnancy, growth periods, and under the conditions of intensive work. Their lack or excess can cause serious health problems [7], [8]. Tolerances for nutrition labelling purposes are important given that it is not possible that foods always contain the exact nutrient levels labelled, due to natural variations and variations from production and during storage. However, the nutrient content of foods should not deviate substantially from labelled values to the extent that such deviations could lead to consumers being misled [9]. Due to their instability and degradation in food, producers add significantly higher concentrations of vitamins to ensure the labelled value until the expiration date. According to the European Union (EU) commission, the acceptable deviation of the declared label value regarding vitamins is +50% and −35% [10].

1.2 Cereal Matrix

Enjoyed throughout the world at the start of the day, a breakfast of fortified cereal with the addition of milk and fruits can be a simple and quick solution to a nutritious meal. Fortified breakfast cereals are also an important source of

nutrition for children, and consumers have come to expect high quality from a variety of cereals and continue to select fortified products over non-fortified products in the marketplace. Breads, especially those made with refined flours, are also usually enriched in order to compensate for any nutritional deficiencies in the cereal used [11][12].

As cereals consist an important part of human diet are usually marketed in their raw grain form (some are frozen or canned) or raw ingredients of various food products. As animal feed, they are consumed mainly by livestock and poultry, which are eventually rendered as meat, dairy, and poultry products for human consumption. Many cereals are used industrially in the production of a wide range of substances, such as glucose, adhesives, oils and alcohols [11].

Cereals are generally of the gramineous family and, in the Food and Agriculture Organization (FAO) concept, refer to crops harvested for dry grain only (Table 1). Crops harvested green for forage, silage or grazing are classified as fodder crops. Also excluded are industrial crops, e.g. broom sorghum (Crude organic materials nes) and sweet sorghum when grown for syrup (Sugar crops nes). For international trade classifications, fresh cereals (other than sweet corn), whether or not suitable for use as fresh vegetables, are classified as cereals. Cereals are identified according to their genus. However, when two or more genera are sown and harvested as a mixture they should be classified and reported as "mixed grains" [12]. The most commonly cultivated cereals are wheat, rice, rye, oats, barley, corn (maize), and sorghum [11]. Cereal products derive either from the processing of grain through one or more mechanical or chemical operations, or from the processing of flour, meal or starch. In the table below are presented the cereal products according to the origin of the cereal [12].

Table 1: Cereals and cereal products [12]

Cereal	Cereal by-products	Short description
Wheat	Flour of wheat, Bran of wheat, Macaroni, Germ of wheat, Bread, Bulgur, Pastry, Starch of wheat, Wheat gluten, Wheat-fermented beverages	Common and durum wheat are the main types. Among common wheat, the main varieties are spring and winter, hard and soft, and red and white. At national level, different varieties should be reported separately, reflecting their different uses. Used mainly for human food.
Rice	Husked rice, Milled rice (Husked), Milled rice, Broken rice, Rice gluten, Starch of rice, Bran of rice, Oil of rice, Cake of rice bran, Flour of rice, Rice-fermented beverages	Rice grain after threshing and winnowing. Also known as rice in the husk and rough rice. Used mainly for human food.
Barley	Pot barley, Pearled barley, Bran of barley, Barley flour and grits, Malt, Malt extract, Beer of barley	Tolerates poorer soils and lower temperatures better than does wheat. Varieties include with husk and without (naked). Used as a livestock feed, for malt and for preparing foods. The roasted grains are a coffee substitute.
Maize	Germ of maize, Flour of maize, Bran of maize, Oil of maize, Cake of maize, Maize gluten, Starch of maize, Beer of maize, White maize, Pop corn	A grain with a high germ content. At national level, hybrid and ordinary maize should be reported separately owing to widely different yields and uses. Used largely for animal feed and commercial starch production.
Rye	Flour of Rye, Bran of Rye	A grain that is tolerant of poor soils, high latitudes and altitudes. Mainly used in making bread, whisky and beer. When fed to livestock, it is generally mixed with other grains.
Oats	Rolled oats, Bran of oats	A plant with open, spreading panicle-bearing large spikelets. Used primarily in breakfast foods. Makes excellent fodder for horses.
Millets	Flour of millets, Bran of	Small-grained cereals that include a large number of different botanical species. Originated by the domestication of wild

	millets, Beer of millets	African grasses in the Nile valley and the Sahel zone, millets were subsequently taken to China and India. These cereals tolerate arid conditions and possess a small, highly nutritious grain that stores well. Used locally, both as a food and as a livestock feed. In all areas where they are cultivated, millets are used in traditional beer brewing. Also used as a feed for birds.
Sorghum	Flour of sorghum, Bran of sorghum, Beer of sorghum	A cereal that has both food and feed uses. Sorghum is a major food grain in most of Africa, where it is also used in traditional beer brewing. It is desirable to report hybrid and other varieties separately.
Buckwheat	Flour of buckwheat, Bran of buckwheat	A minor cereal cultivated primarily in northern regions. Buckwheat is considered a cereal, although it does not belong to the gramineous family.
Quinoa	-	A minor cereal, which tolerates high altitudes, quinoa is cultivated primarily in Andean countries. Used for food and to make chicha, a fermented beverage.
Fonio	Flour of Fonio, Bran of Fonio	A minor cereal of importance only in West Africa where it is eaten in place of rice during famines. The seeds are cooked by steaming the whole grain.
Triticale	Flour of triticale, Bran of triticale	A minor cereal that is a cross between wheat and rye, combining the quality and yield of wheat with the hardiness of rye.
Canary seed	-	Minor cereal normally used as bird feed.
Mixed grain	Flour of mixed grain, Bran of mixed grain	A mixture of cereal species that are sown and harvested together. The mixture wheat/rye is known as muesli, but in trade is usually classified with wheat.
Cereals nes	Flour of Cereals nes, Bran of Cereals nes, Infant food, Wafers, Breakfast cereals,	Other cereal crops that are not identified separately because of their minor relevance at the international level. Because of their

	Cereals preparations, Mixed and Doughs, Food preparations of flour, meal or malt extract, Chemically pure maltose, Other fructose and Syrup, Glucose and Dextrose, Isoglucose, Straw and Husks, Gluten feed and Meal	limited local importance, some countries report cereals under this commodity heading that are classified individually by FAO.

Apart from moisture content and inedible substances such as cellulose, cereal grains contain, along with traces of minerals and vitamins, carbohydrates - mainly starches - (comprising 65-75% of their total weight), as well as proteins (6-12%) and fat (1-5%) [11]. The efficient production of these nutritionally fortified breakfast cereals requires careful formulation and uniformity from batch to batch. Ongoing analytical measurement of nutritional additives and the total micronutrient content in the cereal is one way in which food producers can quantify the quality and consistency of their cereal products. The ability to analyze quickly, accurately, and easily their samples is also key to timely data reporting, allowing real-time batch adjustments to be made and enhancing continuous process control. Food producers should also meet nutritional labelling guidelines which require an accurate assessment of micronutrients for regulatory labelling compliance.

The determination of cereal quality, on the transaction in the organised market, is an indispensable operation to evaluate the cereal product, particularly at the level of collection, only for criteria relative to specific height and, in some cases, to humidity, and its aptitude in storage. Besides, cereals like any other biological product change during their storage when they are badly conserved, causing degradations of quality and loss in quantity [13].

1.3 Vitamins

Vitamin is any of several organic substances that are necessary in small quantities for normal health and growth in higher forms of animal life. Vitamins are distinct in several ways from other biologically important compounds such

as proteins, carbohydrates, and lipids. Although these latter substances also are indispensable for proper bodily functions, almost all of them can be synthesized by animals in adequate quantities. Vitamins, on the other hand, generally cannot be synthesized in amounts sufficient to meet bodily needs and therefore must be obtained from the diet or from some synthetic source. For this reason, vitamins are called essential nutrients [14].

In 1890 a nerve disease (polyneuritis) broke out among a Dutch physician's and pathologist's, Christiaan Eijkman, laboratory chickens. He noticed that the disease was similar to the polyneuritis associated with the nutritional disorder beriberi. In 1912 a Polish scientist, Casimir Funk proposed that the polyneuritis arose because of a lack in the birds' diet of a vital factor (now known to be thiamin) that could be found in rice bran. Funk believed that some human diseases, particularly beriberi, scurvy, and pellagra, also were caused by deficiencies of factors of the same chemical type. As each of these factors had a nitrogen-containing component known as an amine, he called the compounds "vital amines," a term that he later shortened to "vitamines." The final e was dropped later when it was discovered that not all the vitamins contain nitrogen and, therefore, not all are amines [14].

Vitamins vary widely in chemical structure, biological activity and physicochemical properties [15]. Owing to this heterogeneity, the classification as water-soluble (B-complex andC)and fat-soluble (A, D, E, and K) is based on their solubility. Onthe whole, 13 groups are recognized in human nutrition (the B-complexgroups together the vitamins B1, B2, B3, B5, B6, B7, B9, and B12) and each ofthem is composed of several biologically active forms, known as vitamers,which differ in structure, biopotency, and stability [16]–[18].

Vitamins regulate reactions that occur in metabolism, in contrast to other dietary components known as macronutrients (e.g., fats, carbohydrates, proteins), which are the compounds utilized in the reactions regulated by the vitamins [14]. Absence of a vitamin blocks one or more specific metabolic reactions in a cell and eventually may disrupt the metabolic balance within a cell and in the entire organism as well. As a result they play an important role in maintaining blood glucose and are essential for scavenging of free radicals [15], [19].

Vitamins, which are found in all living organisms, either because they are synthesized in the organism or they are acquired from the environment, are not distributed equally throughout nature. Some are absent from certain tissues or species; for example, beta-carotene, which can be converted to vitamin A, is synthesized in plant tissues but not in animal tissues. On the other hand, vitamins A and D_3 (cholecalciferol) occur only in animal tissues. Both plants and animals are important natural vitamin sources for human beings. Since vitamins are not distributed equally in foodstuffs, the more restricted the diet of an individual, the more likely it is that he will lack adequate amounts of one or more vitamins. Food sources of vitamin D are limited, but it can be synthesized in the skin through ultraviolet radiation (from the sun); therefore, with adequate exposure to sunlight, the dietary intake of vitamin D is of little significance [14]. In any case their bioavailability depends on the age, sex, and health status as well as the chemical and physical form of each vitamin [10].

Inadequate intake of a specific vitamin results in a characteristic deficiency disease (hypovitaminosis), the severity of which depends on the degree of the vitamin deprivation. Symptoms may be specific (e.g., functional night blindness of vitamin A deficiency) or nonspecific (e.g., loss of appetite, failure to grow). All symptoms for a specific deficiency disease may not appear; in addition, the nature of the symptoms may vary with the species. Some effects of vitamin deficiencies cannot be reversed by adding the vitamin to the diet, especially if anon-regenerative tissue is damaged (e.g.,corneaof the eye, nerve tissue, calcified bone) has occurred [14].

Supplementary dietary intake of vitamins may result in adverse chronic and acute effects such as osteoporosis and hip fracture which resulted from hypervitaminosis A [20].

The vitamin content of foods is susceptible to losses and vitaminscan be destroyed during technological and cooking processes becauseof their solubility in water. However, some vitamins are more sensitive to additional losses [21], [22]. All vitamins, water-soluble and fat-soluble,are very susceptible to degradation by chemical and physical factors. Water-soluble vitamins are more

labile to temperature and light but fat-soluble vitamins are susceptible to atmospheric oxygen [10].

Historically, food fortification started with milk and its fortificationwith vitamin D. Nowadays, there is an increased interest in nutritionallyrich foods (natural and minimally processed) [17], in particular infant foods, fruit juices and milk [23], and pharmaceutical preparations [24], [25] in order to prevent therisk of nutrient diseases related to vitamin deficiencies such as beriberi, goiter, scurvy, pellagra, neglected tropical diseases (NTDs), rickets, etc, maintain health, restore vitality, control weightand help in disease prevention [17]. The most vulnerablepopulation groups regarding deficiencies are children, athletes, andpregnant and lactating women.

According to World Health Organization (WHO), the fortification strategies are mass, targeted,and market driven. National programs in more than 67 countries financethe fortification process of foods with vitamins to correct thewidespread nutrient losses in processing and storage of products, toenhance nutrient intakes of vitamins, and to attract the consumer's interest [10]. Even though food fortification is cost effective, sometimes manufacturers add higher quantities of the nutrients to ensure its presence at declared levels in the labels because of potential losses until the moment the consumer consumes the products [26]–[29]. To comply with the regulatory requirements for precise labelling and quality assurance on infant foods fortified with vitamins and dietary supplements, reliable analytical methods for the qualitative and quantitative analysis of vitamins in foods are important issues and a challenging task for food manufacturers [20], [25], [30].

1.3.1 Water-soluble vitamins (WSVs)

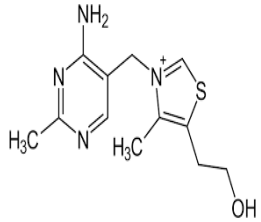
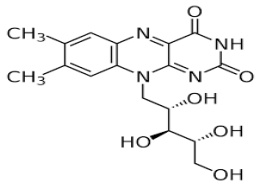
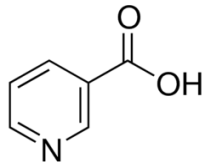
Although the vitamins included in this classification are all water-soluble, the degree to which they dissolve in water is variable. This property influences the route of absorption, their excretion, and their degree of tissue storage and distinguishes them from fat-soluble vitamins, which are handled and stored differently by the body. The active forms and the acceptednomenclature of individual vitamins in each vitamin group are given in the table. The water-soluble

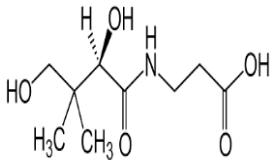
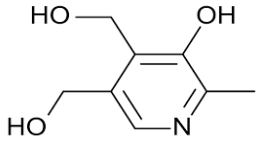
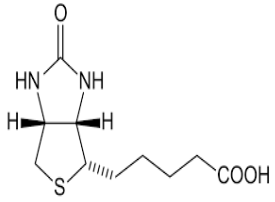
vitamins arevitamin C (ascorbic acid) and the B vitamins, which include thiamine (vitamin B1), riboflavin (vitamin B2), pyridoxine (vitamin B6), niacin (vitamin B3), cyanocobalamin (vitamin B12), folic acid (vitamin B9), pantothenic acid (vitamin B5), and biotin (vitamin B7). These relatively simple molecules contain the elements carbon, hydrogen, and oxygen; some also contain nitrogen, sulphur, or cobalt [14].

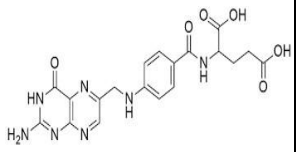
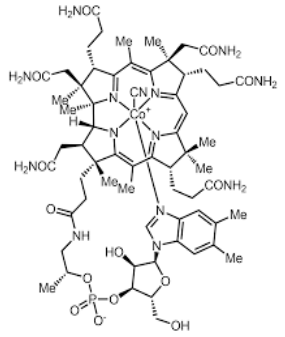
The vitamins of complex B, inactive in their so-called free states, must be activated to their coenzyme forms. After an active coenzyme is formed, it must combine with the proper protein, fats or carbohydrate component before metabolic, enzyme-catalyzed reactions can occur [14].

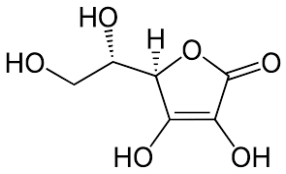
In Table 2 are presented the active forms of each vitamin, their nomenclature, the sources and their biological function.

Table 2: Water soluble vitamins and their properties [31], [32], [41]–[43], [33]–[40]

Vitamin	Structure	Biological function	Commercial availability	Main sources	Recommended daily dose (RDI)
Thiamine (Vitamin B1)		Antioxidant, erythropoietic, mood modulating, and glucose-regulating activities	Thiamine chloride, thiamine hydrochloride, thiamine mononitrate, thiamine monophosphate, thiamine pyrophosphate, and thiamine triphosphate	Yeast, cereal grains, beans, nuts, and meat	1.0-1.5 mg
Rivoflavin (Vitamin B2)		Precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD)	Riboflavin (RF), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), RF hydrochloride and RF 5'-phosphate	Milk, meat, eggs, nuts, enriched flour, and green vegetables	1.1-1.3 mg
Niacin (Vitamin B3)		Effective in lowering low density lipoprotein (LDL) cholesterol and raising high density lipoprotein (HDL) cholesterol, can cause mild-to-moderate serum aminotransferase elevations, component of the coenzymes nicotinamide adenine dinucleotide (NAD) and its phosphate form, NADP	Nicotinic acid, nicotinamide	Greens, meat, poultry, fish, and eggs	35 mg

Pantothenic acid (Vitamin B5)		Component of coenzyme A (CoA), growth factor, essential for various metabolic functions, involved in the synthesis of cholesterol, lipids, neurotransmitters, steroid hormones, and hemoglobin	D-pantothenic acid, dexpanthenol, and calcium pantothenate	Both plants and animals including meat, vegetables, cereal grains, legumes, eggs, and milk	4 mg
Pyridoxine (Vitamin B6)		Coenzyme for synthesis of amino acids, neurotransmitters, sphingolipids, and aminolevulinic acid, cofactor, a human metabolite, a *Saccharomyces cerevisiae* metabolite, an *Escherichia coli* metabolite, and a mouse metabolite	Pyridoxine, pyridoxal, pyridoxamine, pyridoxine 5'-phosphate, pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate	Cereals, beans, vegetables, liver, meat, and eggs	1.2-1.7 mg
Biotin (Vitamin B7)		Important component of enzymes in the body that break down certain substances like fats, carbohydrates, and others	D-(+)-biotin, D-biocytin(ε-N-biotinyl-L-lysine)	Eggs andmilk	-

Folic acid (Vitamin B9)		Involved in producing the genetic material called DNA and in numerous other bodily functions	Folic acid (pteroylglutamic acid), dihydrofolic acid, tetrahydrofolic acid (H4-folic acid), 5-formyl-H4-folic acid, 5-methyl-H4-folic acid, and their derivatives characterized by chains containing 2– 8 glutamic acid residues	Leafy vegetables (such as spinach, broccoli, and lettuce), okra, asparagus, fruits (such as bananas, melons, and lemons) beans, yeast, mushrooms, meat (such as beef liver and kidney), orange juice, and tomato juice	0.4 mg
Cyanocobalamin (Vitamin B12)		Necessary for hematopoiesis, neural metabolism, DNA and RNA production, and carbohydrate, fat, and protein metabolism, improves iron functions in the metabolic cycle and assists folic acid in choline synthesis	Hydroxocobalamin, 5 ′-deoxyadenosylcobalamin (coenzyme B12) , and methylcobalamin	Meat, fish, and dairy products	2.4 mg

L-ascorbic acid (Vitamin C)		Important for the proper development and function of many parts of the body and in maintaining proper immune function	L-Ascorbic acid (AA), L-dehydroascorbic acid (DHAA), D-Isoascorbic acid (D-IAA)	Fresh fruits and vegetables, especially citrus fruits	75-90 mg

1.3.1.1 Vitamin B1 (Thiamine)

Thiamine, also spelled thiamin, also called vitamin B_1, was the first B vitamin to have been identified. In 1926 thiamine was the first vitamin isolated in pure form. Its structure was fully elucidated and the vitamin synthesized in 1936. It carries out its functions in its active form, as a component of coenzyme thiamine pyrophosphate. The thiamine-dependent enzymes are important for the biosynthesis of neurotransmitters and for the production of reducing substances used in oxidant stress defences, as well as for the synthesis of pentoses used as nucleic acid precursors. Thiamine plays a central role in cerebral metabolism [44]–[48].

Thiamine is found abundantly in cereal grains and in certain other seeds. In many countries, white rice and white wheat flour are now fortified with synthetic thiamine. Pork is one of the richest animal sources. The recommended daily intake of thiamine is from 1.0 to 1.4 mg (1 mg = 0.001 gram) for adult humans [49].

Despite long-term thiamine fortification of a variety of common household foods (meats, vegetables, milk products, cereals and whole grains), thiamine deficiency continues to be present even in modern, western societies [50]–[54]. Classically, thiamine deficiency causes the disease known as beriberi, but constitutes really one of several thiamine-deficiency-related conditions, which may occur concurrently, whose manifestations consist of nystagmus, ophthalmoplegia, and ataxia evolving into confusion, retrograde amnesia, cognitive impairment, and confabulation [55]. Key contributors to thiamine deficiency include hospitalisation, moderate to severe alcohol consumption, and improper nutritional intake, e.g. a high carbohydrate load with restricted vitamin and mineral ingestion. Age, as well as co-morbidities, such as cardiac and liver dysfunction, surgery (e.g. bariatric), lactic acidosis, sepsis, trauma and refeeding syndrome (RFS) may also be associated risk factors for thiamine deficiency [50], [56]–[59]. Treatment by thiamine supplementation is beneficial for diagnostic and therapeutic purposes [55].

1.3.1.2 Vitamin B2 (Rivoflavin)

Riboflavin, also called vitamin B2, a yellow, water-soluble organic compound that occurs abundantly in whey (the watery part of milk) and in egg white (Figure 1). An essential nutrient for animals, it can be synthesized by green plants and by mostbacteria and fungi. The greenish yellow fluorescence of whey and egg white is caused by the presence of riboflavin, which was isolated in pure form in 1933 and was first synthesized in 1935 [60].

Figure 1: Rivoflavin solid [60]

Riboflavin functions as part of metabolic systems concerned with the oxidation of carbohydrates and amino acids, the constituents of proteins. Like thiamine (vitamin B1), it is active not in the free form but in more complex compounds known as coenzymes, such as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), or flavoprotein), which participate in oxidation reactions. As a result , riboflavin plays an important role in the citric acid cycle [10].

In addition, vitamin B2 participates in energy production as well as in cellular function, growth, and development [61], [62]. One of the most important characteristics of riboflavin is its photosensitivity as it is easily degraded by light (UV and visible) to biologically inactive products. However, it is stable to heat and oxidation [63].

Riboflavin is widely distributed in both plants and animals, but its abundance varies considerably. Milk, eggs, leafy vegetables, kidney, and liver are good

dietary sources. There is a particular interest in riboflavin-fortified foods, especially for milk, milk products, and beverages because the decrease of vitamin content is a quality marker of vitamin degradation [62]. An adult needs from 1.0 to 1.3 mg (1 mg = 0.001 gram) of vitamin per day [60].

A dietary lack of riboflavin is characterized by variable symptoms that may include reddening of the lips with cracks at the corners of the mouth (cheilosis); inflammation of the tongue (glossitis); ocular disturbances, such as vascularization of the eyeball with eyestrain and abnormal intolerance of light; and a greasy, scaly inflammation of the skin. Some disagreement persists as to the characteristic syndrome of riboflavin deficiency in humans because it tends to be associated with a deficiency of other vitamins, notably niacin [10], [60].

1.3.1.3 Vitamin B3 (Niacin)

Nicotinic acid (NA) and nicotinamide (NM), collectively referred as niacin, are nutritional precursors of the bioactive molecules nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). NAD and NADP are important cofactors for most cellular redox reactions, and as such are essential to maintain cellular metabolism and respiration. NAD also serves as a cosubstrate for a large number of ADP-ribosylation enzymes with varied functions [64], [65].

Vitamin B3 plays an important role in energy metabolism suppressing the expression of inflammatory genes and providing therapeutic benefits in various diseases such as pellagra, which is characterized by dermatitis, dementia, and diarrhea [66], [67].

Like thiamine and riboflavin, niacin functions as part of a coenzyme involved in the metabolism of carbohydrates and acts catalyzing the oxidation of sugar derivatives and other substances [67].

Niacin is the most stable water-soluble vitamin. In aqueous solution, both NA and NM are stable to oxygen, thermal food processing, and light. When heated at 100°C, NA is stable but NM is quantitatively hydrolyzed to NA. Both NA and NM

are used for food fortification, NA in dry products and NM in dry and liquid products [68]–[70].

Niacin, which was identified as a pellagra preventive in 1937, is widely distributed among plants and animals. Lean meat is generally a good source. Approximately 15 mg per day (1 mg = 0.001 gram) of nicotinic acid is required by humans. In the intestinal tract, the amino acid tryptophan can be converted to niacin by bacterial action and thus can serve as a source for part of the required niacin. This explains scientists' early observation that the protein in such foods as eggs and milk, both poor sources of niacin, can nevertheless prevent or cure pellagra [67].

1.3.1.4 Vitamin B5 (Pantothenic Acid)

Pantothenic acid is a water-soluble B-complex vitamin that was identified in 1933, isolated and extracted from liver in 1938, and first synthesized in 1940. Williams coined the name pantothenic acid from the Greek meaning "from everywhere", to indicate its widespread occurrence in foodstuffs [71], [72].

The nature of the bound form was clarified through the discovery and synthesis (1947–50) of the compound pantetheine, which contains pantothenic acid combined with the compound thioethanolamine. Pantetheine is part of two larger compounds (coenzyme A and acyl-carrier protein) that promote the metabolic reactions of fat and alcohol and it is essential for the function of the brain and the synthesis of membranes, hormones, and functional proteins [10], [73], [74].

Chemical forms of vitamin B5, and its derivatives or precursors, are added to foods and beverages, as well as to dietary supplements. Only the D-isomer of pantothenic acid - D-pantothenic acid - possesses biologic activity. Pure D-pantothenic acid can be used as a dietary supplement: it is water-soluble, viscous, and yellow in colour. As D-pantothenic acid is relatively unstable, it can be destroyed by heat, acid, and alkaline conditions. In addition, the more stable calcium pantothenate is the form of vitamin B5 usually found in dietary supplements. It is water-soluble, crystalline, and white in colour. 10 mg of calcium pantothenate is approximately equivalent to 9.2 mg of pure D-pantothenic acid.

The disulphide form of pantothenic acid, pantethine, is also available as a dietary supplement. It is considered the most active form of vitamin because it contains the sulfhydryl-group needed for biological activity in Coenzyme A (CoA). A liquid form of vitamin - dexpanthenol, D-pantothenyl alcohol, D-panthenol, or panthenol - is also available. This is an alcohol pro-vitamin of vitamin (i.e., it is converted into pantothenic acid in the body), which is used primarily as a topical or injected form for cosmetic purposes or wound healing [75].

Vitamin B5 is present in small amounts in all food groups. It can be consumed also as a dietary supplement. Good sources can considered meat, liver, kidney, fruits, fresh vegetables, milk, egg yolk, yeast, whole grains and nuts. During cooking, the vitamin's content is lost only in meat but not in vegetables [10], [76]. There is no a recommended dietary allowance (RDA) for this vitamin due to the existence of insufficient proof. Though, in USA the RDA for adults fluctuates between 5-20 mg per day while 5-10mg per day is probably considered a sufficient amount [76].

Pantothenic acid is most stable under acidic conditions (pH=4.0–5.0) and is unaffected by light and atmospheric oxygen. However, it is susceptible to temperature and is easily degraded under alkaline environments. In the presence of pantethase enzymes, pantothenic acid is hydrolyzed to pantoic acid and β-alanine [77].

In the absence of pantothenic acid, experimental animals fail to grow, develop skin lesions, and frequently show a graying of their hair. Owing to its ubiquitous distribution in foods of both animal and plant origin, pantothenic acid deficiency in humans is rare and its symptoms are reversible [74]. However, when a person is severely malnourished, deficiency of the vitamin appears to contribute to the observed weakness and mental depression, accompanied by vomiting, sleeplessness, and increased susceptibility to infection [10]. Deficiency can be addressed through vitamin supplementation and diet; natural foods, such as sunflower seeds, and certain manufactured food products, such as breakfast cereals, contain sufficient amounts of pantothenic acid to fulfil dietary needs in humans [73].

1.3.1.5 Vitamin B6 (Pyridoxine)

Vitamin B6 is an essential micronutrient for microorganisms and animals. It consists of three derivatives: pyridoxine (or pyridoxol), pyridoxal, and pyridoxamine and their 5′-phosphorylated forms, pyridoxine 5’ -phosphate (PNP), pyridoxal 5’ -phosphate (PLP) and pyridoxamine 5’ -phosphate (PMP). Pyridoxine was first isolated in 1938 and synthesized in 1939. Pyridoxal and pyridoxamine, which were discovered in the 1940s, are responsible for most of the vitamin B_6 activity in animal tissues [10], [78].

Its main biologically active form is a phosphate ester of its aldehyde form, PLP. It plays a major role in many biological pathways, allowing the proper functioning of over 60 enzymes, especially involved in amino acid metabolism including decarboxylation, desamination, transamination and transsulphuration, and hence indirectly of protein. Examples are the metabolism of tryptophan to niacin, methionine to cysteine and glutamic acid to gamma-aminobutyric acid (GABA) [79]. Vitamin B6 has various functions affecting the immune and nervous systems, gluconeogenesis, lipid metabolism, hormone modulation, and gene expression [80]. It is also an efficient singlet oxygen quencher and potential antioxidant [81]. PLP is required for post-embryonic root development, and protects plants from high-salt, ultraviolet rays, osmotic and oxidative stresses [82], [83].

Most bacteria, fungi, and plants possess vitamin B6 biosynthesis pathways, but mammals must be supplied the vitamin in their diet [84]. Different vitamin B6 biosynthetic pathways, referred as de novo biosynthetic pathways, and the salvage pathway, are known. In *E. coli*, PNP is synthesized de novo from the condensation of deoxyxylulose 5-phosphate and 4- hydroxythreonine-4-phosphate, catalyzed by the genesPdxA and PdxJ[85]. A number of other bacteria, plants, and fungi utilize ribose 5-phosphate or ribulose 5-phosphate and dihydroxyacetone phosphate or glyceraldehyde 3-phosphate to synthesize PLP [86], [87].

Vitamin B6 is widely distributed in foodstuffs and is particularly abundant in cereal grains, meats, poultry, fish, legumes, nuts and some fruits and vegetables.

Afteritsabsorption in the intestine, the major part is converted in the liver to pyridoxal-phosphate [78], [88]. The normal adult needs 1–2 mg per day, but requirements are increased during pregnancy [79]. The B6 vitamers are stable in acid but are very photosensitive and especially in UV. The ionization of vitamin B6 depends on the pH and the temperature [10].

No human disease has been caused by the deficiency of vitamin B6 in the diet, although certain metabolic disorders respond to its administration [78]. It is considered that some drugs interact with vitamin B6 reducing its concentration, especially when they are consumed for years [89]and there are substances that promote pyridoxine depletion (isoniazide, cycloserine, hydralazine, phenelzine, penicillamine, theophylline, carbon disulphid). Low levels of vitamin B6 can be found in the elderly, alcoholics, those with HIV infection or diabetes, increasing the risk of cardiovascular diseases and polyneuropathy. In this case, vitamin B6 supplements are advised for children up to 6 years and for adults over 51 years of age [10], [79].

An excess of vitamin B6 can cause nerve damage in the arms and legs, as well as other nervous changes [78]. Nevertheless a high-dose pyridoxine cure is proven to function as an anti-stress strategy [90].

1.3.1.6 Vitamin B7 (Biotin)

Biotin is a nitrogen-containing acid essential for growth and well-being in animals and some microorganisms. Biotin was first identified as a nutritive requirement of yeast. Originally called vitamin H, it was isolated in pure form in 1935.Its structure was established in 1942, after it had been shown to be required by animals [91].

Biotin serves as a coenzyme for 5 carboxylases - acetyl-CoA carboxylases 1 and 2, propionyl-CoA carboxylase (PCC), 3-methylcrotonyl-CoA carboxylase (MCC), and pyruvate carboxylase [92] - to important metabolic pathways such as amino acids, fatty acids, and cholesterol catabolism, gluconeogenesis and fatty acid metabolism [10]. Biotin also regulates the catabolic enzyme PCC at the posttranscriptional level whereas the holo-carboxylase synthetase is regulated at the transcriptional level. Aside from its role in the regulation of gene expression of

carboxylases, biotin has been implicated in the induction of the receptor for the asialoglycoprotein, glycolytic enzymes and of egg yolk biotin binding proteins [93].

Vitamin B7 can be synthesized by intestinal bacteria, supplying enough to the body to cover its requirements under most circumstances [10]. The AI (Adequate Intake) for biotin is 30 µg per day in men, women and pregnant women, and increases to 35 µg per day for lactating women [94].

In foods, vitamin B7 is available in either free or bound form. It is widely distributed in various foods such as soybeans, almonds, vegetables, walnuts, bean sprouts, grains, milk, and egg yolk. However, fruits and meats, except for beef liver, are poor sources [10], [92].

Biotin is relatively stable compared to other water-soluble vitamins. In the dry form, it is stable to heat and atmospheric oxygen, but is labile to UV light. In solution, it is stable up to 100°C at pH 4.0-9.0 [77]. This is the reason why biotin deficiency is not common in humans. Evidence for the necessity of biotin appeared with the discovery in 1927 that the addition of uncooked egg white to a diet that is otherwise adequate produces toxicity and disease. This fact occurs because egg white contains a specific protein, avidin,that combines with biotin and thus prevents its absorption. In practice, biotin deficiency is generally associated with prolonged parenteral nutrition, consumption of large quantities of avidin, usually in the form of raw eggs, severe malnutrition and, inherited metabolic disorders. In humans, there are autosomal recessive disorders of biotin metabolism that result from the disruption of the activity of biotinidase or holo-carboxylase synthetase, which catalyze reactions in the process of the degradation of carboxylases and of their conversion to their active form, respectively [93].

Symptoms include dermatitis, conjunctivitis, alopecia,ataxia, hypotonia, ketolactic acidosis/organic aciduria, seizures, skin infection, thinning hair, skin rashes around the eyes, nose, and mouth, impaired immune function and developmental delay in infants and children. Evidence suggests that biotin catabolism is accelerated in pregnancy and that about half of pregnant women in the United

States are marginally biotin deficient [92]. Likewise, biotin deficiency might be encountered in smokers, users of certain anticonvulsants, and after consumption of pharmacological doses of lipoic acid.

No tolerable upper intake has been specified in the dietary reference intakes [95], [96].

1.3.1.7 Vitamin B9 (Folic Acid)

Folic acid is also known as vitamin B9, pteroylglutamic acid, folate, or folacin. It is essential in animals and plants for the synthesis of nucleic acids.Folic acid is the synthetic form of B9 found in supplements and fortified foods, while folate occurs naturally in foods [97]. Folic acid was named from the same root word for foliage, as it was found to be abundant in leafy green vegetables [36], [98].

Folate is a group of related compounds, occurring as food folate and folic acid, with the same structure but different chain length and state of reduction. There are 150 folate vitamers and the fully oxidized monoglutamate form of the vitamin, which is a synthetic compound, is usually used in dietary supplements and fortified foods due to its stability compared to the natural vitamers [99]. Folates and folic acid are more stable under dry conditions and if they are protected from light and oxygen [68]–[70], [98].

The physiological properties of folic acid were discovered in the 1930s when it was observed that megaloblastic anemia could be treated by the addition of yeast or liver extracts to patient diets [100]. The active ingredient of the extracts was also found to be an essential growth factor for the bacterium *Lactobacillus casei* and *Streptococcus faecalis*. The development of bioassays based on the growth of these organisms permitted the isolation and ultimately the structural identification of folic acid [101]. It was finally isolated from liver cells in 1943 [98].

Folate exists in several oxidative states (Figure 2), each of which functions as cofactor in metabolism. Intracellular reduction of the pyrazine portion of the pteridine ring leads to the formation of dihydrofolate followed by tetrahydrofolate. Tetrahydrofolate functions as the coenzyme in the utilization of single carbon units.

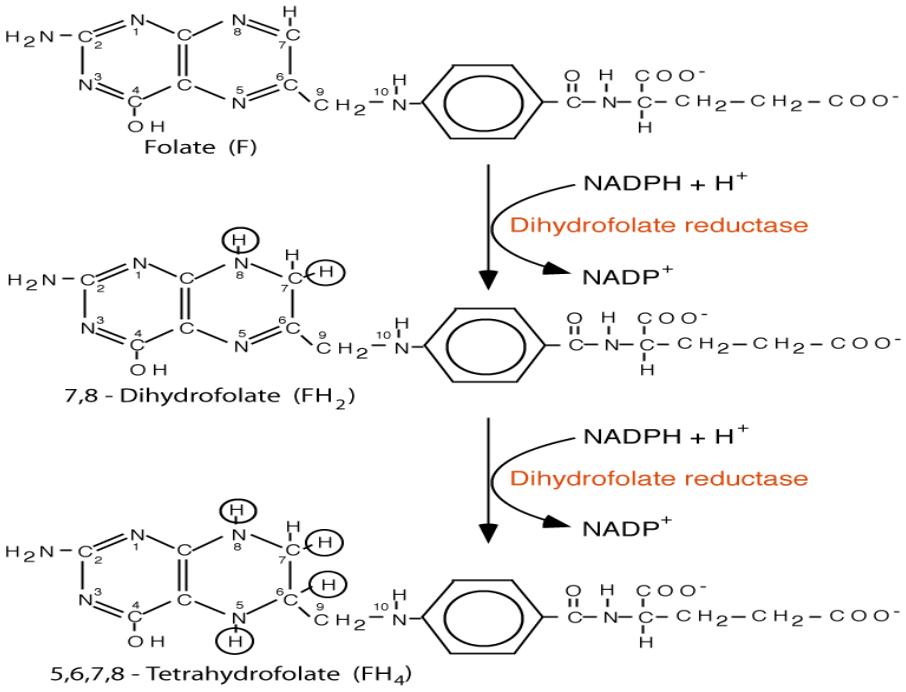


Figure 2: Folic acid and its oxidative states [36]

Humans and animals are incapable of de novo synthesis of folate, in contrast to plants and bacterium, or the storage of appreciable long-term supplies. Therefore, we are dependent on a frequent and adequate supply in our diets. Folate deficiency develops relatively fast with respect to other nutrients such as cobalamin. The absorption of folate is intricately linked to its distribution and its metabolism.A deficiency of folic acid can occur in any age group, albeit for many different reasons. Alterations that can result in folate deficiency can occur at the level of both absorption and metabolism. Some of these disturbances are inherited disorders [36].

It is also crucial for proper brain function and mental and emotional health. It helps the production of DNA and RNA, the body's genetic material, especially when cells and tissues are growing rapidly, such as during infancy, adolescence, and pregnancy. Getting enough folic acid before and during pregnancy may prevent major birth defects of baby's brain or spine [97], [102]–[105]. Folic acid works closely with vitamin B12 in making red blood cells and helps iron function properly in the body. The metabolisms of folic acid and vitamin B12 are intimately linked, such that deficiency of either vitamin leads to disease. Vitamin B9 works

with vitamins B6 and B12 and other nutrients in controlling the blood levels of the amino acid homocysteine.

Folic acid deficiency can cause megaloblastic anemia and birth defects. Megaloblastic anemia is described as presence of large-sized red blood cells than normal. It results from the inhibition of DNA synthesis within red blood cell production. 5-methyl tetrahydrofolate can only be metabolized by methionine synthase; so, lack of folate coenzyme will lead to impaired red blood cells. Since DNA synthesis becomes impaired, the cell cycle cannot progress and cells continue to grow without division, which presents as macrocytosis [36], [106].

Folate has also been implicated in the development of cancer, especially cancer of the colorectum, through two principal mechanisms. Folate deficiency, by reducing intracellular S-adenosylmethionine (SAM), can alter cytosine methylation in DNA, leading to inappropriate activation of proto-oncogenes and induction of malignant transformation. Alternatively, folic acid is crucial for normal DNA synthesis and repair. Folate deficiency may cause an imbalance in DNA precursors, uracil misincorporation into DNA, and chromosome breakage [107].

The linking between B9 and B12 metabolisms leads to the fact that the neurologic manifestations of folate deficiency overlap with those of vitamin's B12 causing cognitive impairment, dementia, depression [108], and, less commonly, peripheral neuropathy and subacute combined degeneration of the spinal cord. In both deficiency states there is often dissociation between the neuropsychiatric and the hematologic complications [109].

High levels of homocysteine are associated with heart disease, although some researchers are not sure whether homocysteine is a cause of heart disease or just a marker that indicates the presence of heart disease [110].

The daily recommendations for dietary folic acid are 400 mcg (RDA) for adults, while 600 mcg (RDA) for pregnant women [111]. Folate is naturally available in some foods (legumes, leafy green vegetables, citrus fruits, poultry, egg yolks and juices), added to others (fortified products, especially grain and cereal products [112]), and available as a dietary supplement.

In addition folic acid can prevent macrocytic anemia and congenital abnormalities known as NTDs. For this reason, it is important to improve folate intakes with pharmacological supplementation or fortification of foods [113], [114]. Fortification of many cereal-food products became mandatory in the United States on January 1, 1998, and the Canadian milling industry started fortification early in 1997, to meet the U.S. requirements for imported flour [115]. On November 11, 1998, fortification with folic acid of all types of white flour, enriched pasta, and cornmeal became mandatory in Canada [116]. In other countries like Spain, folic acid fortification is allowed and as a result the food industry takes advantage of producing a wide variety of folic acid-fortified milk and milk products, baby foods and non-alcoholic beverages [117].

However, there is scientific evidence that high intakes of folic acid can create adverse effects, mainly in children and the elderly, as it suppresses the development of early lesions in normal tissues and in turn increases the progression of neoplastic cells. Thus, widespread folic acid fortification is limited in many countries [118].

1.3.1.8 Vitamin B12 (Cyanocobalamin)

B12 has the largest and most complex chemical structure of all vitamins [119]. It has been named cobalamin because it contains the rare element cobalt in its chemical structure. Cobalamin belongs to a group of cobaltcontaining compounds known as corrinoids that contain a specific corrin ring. Vitamin B12 was isolated in 1948 as an antipernicious factor [120].

Methylcobalamin and adenosylcobalamin are the biologically active forms of B12. Other forms such as hydroxocobalamin (or aquacobalamin) and industrially produced and used in most supplements cyanocobalamins must be metabolized to the two active forms in order to be used in human cells [121], [122]. A specific R-group attached to the cobalt element is what differentiates chemically the forms of B12. Cyanocobalamin is the most stable vitamer. It is susceptible to degradation in strongly alkaline or acidic solutions, and to UV-visible light [10].

In comparison with other B vitamins, B12 is not synthesized by animals, fungi, or plants. Exclusively, microorganisms (mainly anaerobes) or archaebacteria in the

presence of cobalt are able to produce vitamin B12 [123], [124]. Its synthesis is more complex than that of most other small molecules in nature and involves 30 or more steps [10].

The B12 synthesized in the stomach is absorbed in the intestine, transferred into the blood and stored in the liver and muscles of the animal or secreted into the milk, which other animals and humans eat. Although the intestinal flora of humans is able to synthesize vitamin B12, humans are not able to absorb it because of the complexity of the process [125]. Therefore, vitamin B12 consumed through food and milk is the most important source of B12 for increasing serum B12 levels [126]–[128]. Along with milk, other animal foods (i.e., meat, egg, fish, and shellfish) but not plant foods are considered to be the major dietary sources of vitamin B12 [121]. Tempeh [129], [130], nori [131], [132], *Chlorella*extracts [133] and mushrooms [134] are some plant origin sources of vitamin B12.

The daily Western diet contains around 5 to 30 µg of vitamin B12 daily, with around 10 to 30% being destroyed by cooking [135]. The UK government recommends a daily intake of 1.5 µg of vitamin B12, with the European Union recommending 1 µg and the United States and Japan recommending 2.4 µg. Body storage is relatively high, about 1-5 mg, while *Bor et al.* reported that a daily vitamin B12 intake of 6 µg appears to be sufficient to maintain a steady-state concentration of plasma vitamin B12 and vitamin B12– related metabolic markers [127]. Therefore deficiency from diminished intake or absorption may not manifest for several years after the depletion of stores[136]–[138].

Deficiency can manifest in different groups and periods with high requirements, such as during growth in children and adolescence or in pregnancy. Certain groups may have reduced intake, such as those with poor nutrition, older people, or people who adhere to a vegan or vegetarian diet [139].

B12 (methylcobalamin) is a coenzyme involved in the transfer of a methyl group in a methionine synthase-requiring reaction that converts homocysteine to methionine, which after that biosynthesizes homocysteine again (Figure 3).

Insufficiency of vitamin B12 causes disruption of the cycle and intracellular accumulation of homocysteine, so its blood levels are raised. Raised blood levels of homocysteine constitute a risk factor for vascular disease [140]–[142] and a predictive marker for cognitive decline in healthy older people [143].

In this reaction B12 activates folate, which is essential in DNA synthesis and thus B12 deficiency affects nucleic acid (DNA) synthesis.Since methionine is needed for the synthesis of myelin, a coating of the nerve pathways, B12 deficiency may result in inadequate myelin synthesis, maintenance, and repair and may impair nerve transmission [122].

Adenosylcobalamin is also essential in converting methylmalonic acid to succinyl-CoA through L-methylmalonyl-CoA, a result of an action of another enzyme methylmalonyl-CoA mutase (Figure 3) [122], [144], [145]. In this occasion, vitamin B12 deficiency leads to increased levels of serum methylmalonic acid [142].

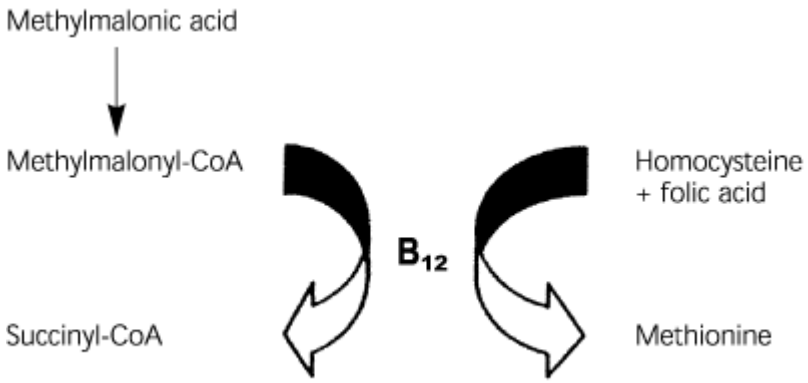


Figure 3: Deficiency of vitamin B12 or folic acid can lead to increased homocysteine levels [146]

Vitamin B12 deficiency also has been linked to psychiatric disorders, including impaired memory, irritability, depression, dementia and, rarely, psychosis [147], [148].

The diagnosis is usually clear in the presence of typical neuropsychiatric syndromes associated with megaloblastic anaemia or macrocytosis and a low

serum concentration of vitamin B12 [149]. Moreover, elevated serum homocysteine levels are frequently found in healthy older people.

Vegetarians and vegans who restrict or eliminate consumption of animal-source foods are at higher risk for developing a B12 deficiency [150]. As the absorption of the vitamin may decline with age, the Food Protection Program recommended that older people, the ones following a restricted diet, as well as, women during pregnancy or lactation should attain their need of this vitamin from supplements or fortified foods (usually breakfast cereals [10], [139], [151].

Finally, it is commonly accepted belief that B12 does not appear to have any toxic side effects even when mega doses of B12 are used as supplements, in parenteral administrations, or in injections [150].

1.3.1.9 Vitamin C (L-ascorbic Acid)

Vitamin C, also ascorbic acid, abbreviated as AA,isprobably the most commonly used vitamin. It is highly soluble in water and functions as an effective reductant. First isolated in 1928, vitamin C was identified as the curative agent for scurvy in 1932 [152], [153]. Ascorbic acid is synthesized by all plants and most animals [154]. It is a vitamin for humans because the gene for gulonolactone oxidase, the terminal enzyme in the AA synthesis pathway has undergone mutations that make it non-functional [155]. It can be found in foods in two vitamers, L-ascorbic acid, a strong reducing agent, and its oxidation product, dehydro-L-ascorbic acid (DHAA). A stereoisomer, isoascorbic acid(IAA), does not occur naturally in foods, but is added as an antioxidant during processing procedures [156].

The adsorption of vitamin C from the dietary sources depends on the facilitated diffusion and a saturable-substrate transport mechanism involving the ascorbate-specific transporters, which saturation and low expression (induced by substrate downregulation) control the effective serum vitamin C concentration [157].

Since humans themselves are not capable of synthesizing it, a minimal daily intake of food origin is therefore necessary and it comesmainly from fresh fruits and vegetables. Milk, meat and fish contain only a very small amount (0 to 2 mg

per100 g). The minimum need to prevent scurvy is 10 mg of ascorbic acid per day and the sufficient amount comes to 350 mg [158].

Vitamin C is one of the most unstable vitamins and for this reason is used as an indicator of overall vitamin stability in foods. DHAA is less stable than AA. It is susceptible to oxygen, elevated temperatures, trace metals such as copper or iron, alkaline pH, ultraviolet light, and oxidizing enzymes [159]–[161].

Chemically, vitamin C is an electron donor, or reducing agent.As electrons from vitamin C can reduce oxidized species, or oxidants, vitamin C is often termed as an antioxidant [162]. Actually, it is one of the four antioxidants in the diet, along with vitamin E, beta-carotene and selenium.It participates in the degradation of oxygenated free radicals, providing protection against toxic agents for the cell [163]. It also functions as a coenzyme of oxidation enzymes such as proline hydroxylase, lysine hydroxylase, 4-hydroxyphenylpyruvate dioxygenase, dopamine-β-hydroxylase, tryptophan hydroxylase, and γbutyrobetaine hydroxylase [164], [165]. Through these enzymatic reactions, AA is involved in the metabolism of neurotransmitters, lipids, and collagen. Its role in the synthesis of collagenis important and its deficiency induces an alteration of the structure collagen. This explains the clinical manifestations of scurvy: altered dentin formation and loss of teeth, damage of the vascular wall and purpura with hemorrhagic syndrome, edema, skin damage due to keratin damage, bone changes due to inability of osteoblasts to form the osteoid border in children [166].

The vitamin also boosts the immune system [152], has a role of cofactor in the synthesis of catecholamines, promotes the absorption of non-heme iron (reduction of ferric ions to ferrous and ferric ion) and plays a role in the mobilization of iron from one form to another (circulating iron bound to siderophilin and reserve iron bound to ferritin) [163]. Finally, the inability of the vitamin to maintain high levels in serum, has a serious health impact on the progression of degenerative diseases, such as cancer and cardiovascular disease (CVD) [167].

Pathologies and certain situationsthatfavour the deficiency of AA are some psychiatric disorders (psychosis, anorexia nervosa), cancer [168], AIDS, intestinal pathologies such as Crohn's disease [169], Whipple's disease [170], and celiac disease [171].

The population groups that are the most vulnerable in vitamin C deficiency is the subject of many studies. The male predominance of hypovitaminosis C is also noted by Jacob et al. [172], but Johnston et al. [173] finds no difference between two sexes. Tobacco decreases absorption and increases catabolism of vitamin C with a daily turnover of vitamin C40% higher for heavy smokers (>20 cig/day) than among non-smokers [174]. Schectman et al. [175] shows that smokers have three times more deficiency in vitamin C, and considers that their daily intake should be greater than 200 mg per day. The influence of tobacco is not found by *Lowik et al.* [176] but a very selected population of older women was studies.

1.3.2 Fat-soluble vitamins

Fat soluble vitamins constitute the other category of the vitamins and they are classed as four groups of compounds, specifically A, D, E and K. They are involved in metabolic reactions of human body’s structural substitutes and for their adequate absorption, the existence of specific amount of lipids in the diet is necessary [177], [178]. Unlike the water- soluble vitamins, they can be stored in the body for longer time periods, so they are required in smaller amounts [177], [179].

Some of the fat-soluble vitamins form part of the structure of biological membranes or assist in maintaining the integrity (and therefore, indirectly, the function) of membranes. Some fat-soluble vitamins also may function at genetic level by controlling the synthesis of certain enzymes [14].

Their determination is tough, due to the complexity of the food matrices and their susceptibility to elevated temperatures, light, oxygen and extreme pH values [180]. During the sample preparation, antioxidants have to be used in order to avoid the oxidative degradation of the vitamins [181], as well as mainly organic solvents for their extraction [182].

CHAPTER 2

ANALYTICAL METHODOLOGIES AND TECHNIQUES

2.1 Analytical methodologies

The widespread use of vitamins created the necessity to develop rapid, sensitive and reliable analytical methods for their determination in fortified or non-fortified food. An overview of the analytical methodologies developed for the simultaneous determination of the water soluble vitamins in food matrices using liquid chromatographic techniques is presented in Table 3.

2.1.1 Sample preparation

Sample preparation is the process which includes the isolation and/or pre-concentration of compounds of interest from various matrices, the removal of any matrix interferences that may affect the detection system. Even with the advances in the development of highly efficient analytical instrumentation for their final determination, sample preparation is a vital part of the analytical procedure and essential for obtaining accurate quantitative results and maintaining instrument performance. The extraction of the analytes from the matrix is the most significant step in a typical sample preparation technique.

2.1.1.1 Sample extraction techniques: Liquid-Liquid extraction (LLE)

Liquid extraction is one of the most popular sample treatment techniques. To obtain optimal results, the selected extraction solvent has to be efficient for the target compounds, whereas the extraction of matrix constituents has to limited in order to prevent excessive matrix effects (ME). The selection of the solvent therefore depends not only on the target compounds, but also on the matrix.

Simple extraction with aqueous buffers (e.g. McIlvaine buffer or succinate buffer) is advantageous for highly polar residues as they reduce nonpolar matrix components (e.g. lipids) and the extracts can be enriched on reversed phase

SPE [183]–[187]. The disadvantage of this extraction is that strongly protein-bound residues are not fully extracted and polar matrix components are co-extracted.

In general, the majority of methods employs more efficient organic solvents as extracting agents. Methanol (MeOH) and acetonitrile (ACN) are more adequate as extraction solvents as they can simultaneously precipitate the proteins and extract the target analytes.

LLE with an organic solvent is a widely applied extraction procedure in vitamins' analysis, supported by a variety of purified solvents or theirmixturesand with great solubility and selectivity ranges. The required instrumentation is simple and in many occasions the organic solvents are totally compatible with the mobile phase [188]. In this category of extraction, saponification, enzymatic hydrolysis, alkoholysis and direct extraction with an organic solvent are all involved.

2.2 Instrumental analysis

2.2.1 Liquid Chromatography (LC)

The separation principle of LC is based on the distribution of the analyte (sample) between a mobile phase (eluent) and a stationary phase (packing material of the column). Depending on the chemical structure of the analyte, the molecules are retarded while passing the stationary phase. The specific intermolecular interactions between the molecules of a sample and the packing material define their time "on-column". Hence, different constituents of a sample are eluted at different times. Thereby, the separation of the sample's ingredients is achieved. A detection unit (e.g. UV detector) recognizes the analytes after leaving the column. The signals are converted and recorded by a data management system (computer software) and then shown in a chromatogram. After passing the detector unit, the mobile phase can be subjected to additional detector units (a fraction collection unit) or to waste (Figure 4). In general, aHigh Pressure Liquid Chromatography (HPLC) system contains the following modules: a solvent reservoir, a pump, an injection valve, a column, a detector unit and a data processing unit. The solvent (eluent) is delivered by a pump at high pressure and constant speed through the system. To keep the drift and noise of the detector

signal as low as possible, a constant and pulseless flow from the pump is crucial. The analyte (sample) is provided to the eluent by the injection valve [189].

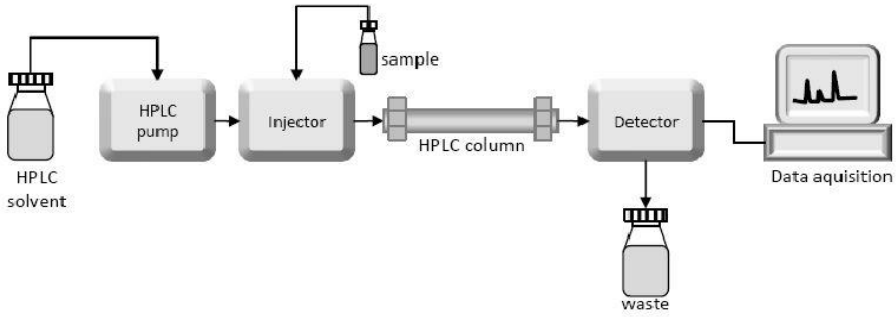


Figure 4: HPLC system components [190]

In chromatography, stationary phase is either a porous solid material with an activated surface and amicroparticulate phase or a thin film of liquid coated on a solid substrate or on a column walls [191].

The stationary phase usually consists of hydrocarbon chains chemically bonded to a filler of silica particles. The silicon grid consists of silicon atoms which are connected to each other by oxygen bridges and on the surface of the grid there are free silanol groups -SiOH. The reaction of silanolic groups with an organochlorosilane results in a chemically bound static phase. Usually the R group is octadecyl (C18), and the long hydrocarbon chains are aligned parallel to each other and perpendicular to the surface of the particle, giving a brush structure. It is often followed by a reaction with chlorotrimethylsilane to cover the free silanol groups (endcapping), which did not react due to stereochemical inhibition by the R group, and which attribute undesired polarity to the stationary phase surface [192], [193].

Polar solvents were used in Reversed-phase Liquid Chromatography (RP-LC). In particular, the mobile phase is usually consisted of mixtures of water or buffers with various water-miscible organic solvents, such as methanol, acetonitrile, dimethylformamide, dioxane and tetrahydrofuran. The composition of the mobile phase throughout the analysis can be kept constant, known as isocratic elution, or it changes over time, and called gradient [193].

2.2.2 Mass Spectrometry (MS)

Mass spectrometry is a powerful analytical technique used to quantify known materials, to identify unknown compounds within a sample, and to elucidate the structure and chemical properties of different molecules. The complete process involves the conversion of the sample into gaseous ions, with or without fragmentation, which are then characterized by their mass to charge ratios (m/z) and relative abundances. This technique basically studies the effect of ionizing energy on molecules. It depends upon chemical reactions in the gas phase in which sample molecules are consumed during the formation of ionic and neutral species.

2.2.2.1 Basic principle

A mass spectrometer generates multiple ions from the sample under investigation, it then separates them according to their specific mass-to-charge ratio (m/z), and then records the relative abundance of each ion type. The first step in the mass spectrometric analysis of compounds is the production of gas phase ions of the compound, basically by electron ionization. This molecular ion undergoes fragmentation. Each primary product ion derived from the molecular ion, in turn, undergoes fragmentation, and so on. The ions are separated in the mass spectrometer according to their mass- to-charge ratio (m/z), and are detected in proportion to their abundance. A mass spectrum of the molecule is thus produced. It displays the result in the form of a plot of ion abundance versus mass-to-charge ratio. Ions provide information concerning the nature and the structure of their precursor molecule. In the spectrum of a pure compound, the molecular ion, if present, appears at the highest value of m/z (followed by ions containing heavier isotopes) and gives the molecular mass of the compound.

2.2.2.2 Instrumentation

The instrument consists of three major components (Figure 5):

1. Ion Source: For producing gaseous ions from the substance being studied.
2. Analyzer: For resolving the ions into their characteristics mass components according to their mass-to-charge ratio.

3. Detector System: For detecting the ions and recording the relative abundance of each of the resolved ionic species. In addition, a sample introduction system is necessary to admit the samples to be studied to the ion source while maintaining the high vacuum requirements (~10-6 to 10-8 mm of mercury) of the technique. A computer is required to control the instrument, acquire and manipulate data, and compare spectra to reference libraries.

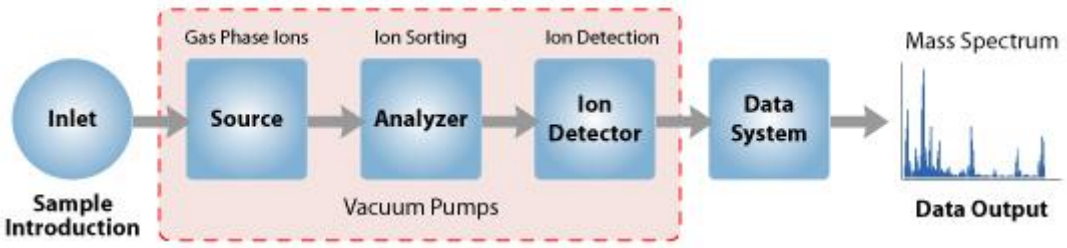


Figure 5: Components of a mass spectrometer [194]

With all the above components, a mass spectrometer should always perform the following processes. It produces ions from the sample in the ionization source, separates these ions according to their mass-to-charge ratio in the mass analyzer and eventually, fragments the selected ions and analyzes the fragments in a second analyzer. Then it detects the ions emerging from the last analyzer and measures their abundance with the detector that converts the ions into electrical signals. Finally, it processes the signals from the detector that are transmitted to the computer and controls the instrument using feedback.

2.2.3 Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS)

Although a number of analytical techniques such as microbiological assays, 1H NMR spectrometry [195], reverse phase TLC [196], electrophoresis [195], and cyclic voltammetry [197] are available. LC is still considered a valid reference technique to analyze all types of vitamins [198], [199].

A great advantage of chromatographic techniques lies on the possibility of performing quantitative multianalyte determinations. The sensitivity of performing trace analyses depends on the coupled detection system. Gas chromatography (GC), using capillary columns, is characterized by a high resolving power, but for

the analysis of polar, non-volatile and heat-sensitive compounds, such as water-soluble vitamins. LC is the most suitable technique.

Moreover, LC presents fewer limitations regarding the molecular weight of the molecule that has to be analyzed; this could be particularly advantageous for the direct analysis of high molecular weight vitamers, such as cobalamins, polyglutamate forms, FAD, and CoA [200].

LC-MS techniques provide a universal approach applicable to all the vitamins and this is the reason why this technique has to be selected for the analysis of both fat- and water-soluble vitamins.

The combination of atmospheric pressure ionization tandem mass spectrometry (API-MS/MS), with LC and UPLC is currently the most frequently used technique. The most used API are atmospheric pressure chemical ionization (APCI), and electrospray ionization (ESI). For compounds of moderate to high polarity, ESI constitutes the most adequate ionization technique.

Among the different mass analyzers usually applied for target analysis, triple quadrupole (QqQ) is the most widely used for measuring and quantifying vitamins. Hybrid quadrupole-linear ion trap (Q-LIT) system combines fully functional quadrupole and linear ion trap-MS within the same instrument and thus, apart from great sensitivity, is capable of producing MS^n spectral information, useful for structure elucidation. Q-LIT has been used in fewer applications than simple triple quadruple formats.

However, a recent trend towards the high-resolution mass spectrometry (HRMS; i.e., time-of-flight, TOF; Orbitrap; Fourier Transform-Ion Cyclotron Resonance, FT–ICR) is undoubtedly observed. High Resolution mass analyzers and hybrid mass analyzers, such as Q-TOF and LIT-Orbitrap, open a new era in food analysis together with holistic sample preparation and retrospective analysis. Due to their high resolving power, mass accuracy, fragmentation and isotopic pattern elucidation can provide tentative identification of non-target and unknown compounds in food samples. Full scan acquisition mode and MS^n mode are useful tools of these new generation instruments.

The main source of analytical problems encountered by LC-MS users is related to matrix effect problems, particularly when studying complex samples, such as food. It represents certainly one of the main sources of pitfall for the analyst, affecting many aspects of the method performance, such as detection capability, repeatability and accuracy. Matrix effect mainly appears as ion suppression and it corresponds to the decrease of the evaporation efficiency of the ions of the analyte due to competition effects with co-extracted and co-eluted matrix components. Another proposed mechanism is the competition between analytes and interfering compounds regarding the maximal ionization efficiency of the technique [201]–[203]. Much less frequently and by a process not yet fully understood, the presence of endogenous compounds in the nanodroplets of the electrosprayed solution can result in an increased ion signals for the analytes compared to those of a reference standard solution.

To overcome matrix effects when quantifying, two practicable approaches can be used. The use of adequate isotope-labelled internal standards and/or analyte quantitation by matrix-matched calibration standards should eliminate the analytical systematic errors (bias) caused by ion suppression or ion enhancement [204].

The water-soluble vitamins can be eluted from RP columns with low concentrations of aqueous methanol or acetonitrile and analyzed with detectors such as UV/Vis, fluorescence, PDA, mass spectrometry etc. Among the various types of detectors, the UV detectors have higher sensitivity, and enhance the compatibility of gradients but the magnitude of response depends on the molarity of analytes [198]. Studies have proven that only LC-ESI-MS/MS methods are capable of separating simultaneously all the water-soluble vitamins [17]. Another advantage of UPLC-MS/MS is that does not require complex samples clean-up procedures. Water-soluble vitamins have a diverse structure and chemical properties and most of them are polar compounds with less retention in RP chromatography [205], [206]. UPLC-MS/MS is very selective and sensitive method which can determine even samples with low concentration [206].

2.3 Literature Review

A great number of methods have been developed for the determination of fat and water-soluble vitamins in various food matrices, biological samples and pharmaceutical preparations, especially multivitamin dietary supplements. A valuable source of vitamins for our everyday energy supplementation is found in breakfast cereal matrices and cereal bars. Nevertheless, there are a few methods developed for the determination of vitamins in pure cereal matrices, and not in infant formulas (Table 3).

Table 3: Literature review

Matrix	Analytes	Conditions of analysis	Sample preparations	Reference
Ricebean	B1, C, B3(acid), B5, B6, B9, B2	HPLC-UV/prontosil120-5 C18 AQ column with aguard column/A: acetonitrile,B: 50 mm PDHO buffer (pH 2.85)(potassium di-hydrogen orthophosphate anhydrous)/gradient elution	Dissolution in 0.1 M sodium acetate buffer (4.8)/ boiling water bath for 10min/ incubation at 37 °C in water bath for 4 h/SPE with Gracepure SPE C18 Low cartridges	[198]
Infant formulas and related nutritionals	B1,B2, B3, B5, B6, B7,B9	LC-ESI-MS/MS / Waters Acquity BEH C18 column (2.1 mmx100 mm, 1.7µm)/(A) 20 mm ammonium formate and (B) methanol/gradient elution	Extraction with 750 µL of 1% glacial acid in methanol/centrifugation/ dilution of 100 µL of the supernatant with 20 ml of 50 mM ammonium formate/filtered through a 0.45mm syringe filters	[199]
Maize flour, green and golden kiwi and tomato pulp	B1, B2, B3, B5, B6, B7, B9, B12,C	LC-ESI-MS/MS/lltima C18 column (25 cmx4.6 mm,5mm), with a guard column /(A) acetonitrile (B) water (both acidified with 5mmol/L formic acid)/gradient elution	Samples were mixed with 15 mg of BHT/SPE with C-18-bonded silica and glass cartridges	[205]
Infantformula	B1, B2, B3,B5, B6, B7, B9, B12, C	UPLC-ESI-MS/MS/ Waters ACQUITY UPLC BEH Shield RP18 column (2.1 mm×100 mm, 1.7 µm)/(A) 10 mm aqueous ammonium acetate and (B) methanol/gradient elution	Extraction with 10 mM aq. ammonium acetate in ultrasonic bath for 15min/ addition of 10 ml of chloroform and collection and filtration of the supernatant	[206]
Multivitamintablets	B1, B2, B3, B5, B6, B7, B9, C, A, D3, E	Capillary electrokinetic chromatography with polymeric micelle as	Extraction with 40ml of 1% w/v copolymer P(SPA-co-MAA)inultrasonic bath for	[195]

		pseydostationary phase	20min/centrifugation /filtration of supernatant through 0.45μm nylon filter	
Breakfastcerealmatrix	B1, B2, B3, B6, B9, C	HILIC-UV, FLD and coulometric detection/ZORBAX HILIC Plus silica column (100 mm × 4.6 mm, 3.5 μm)/(A)10mm ammonium acetate(pH5.0) in water:acetonitrile (95:5, v:v), (B) 10mm ammonium acetate(pH5.0) in acetonitrile:water (95:5, v:v)/gradient elution	Mineral acid hydrolysis with 4ml of 0.1M HCl followed by enzymatic treatment with either 20mg (48U) papain and 12.5mg (700U) claradiastase, or acetate buffer/digestion at 37oC for 16h and treatment 100oC for 4min/centrifugation/1μL of the supernatant mixed with 3mL of acetonitrile/centrifugation/filtration through a 0.2μm nylon filter membrane (for B1, B2, B3, B6, B9) extraction with 4mL of 25:75(v:v) of methanol:0.3% meta-phosphoric acid including 200mg/L DTT/centrifugation/1μL of the supernatant mixed with 3mL of acetonitrile/centrifugation/filtration through a 0.2μm nylon filter membrane (for C)	[207]
Mineralfeeds and premixes	B1, B2, B3, B5, B6, B9	Ion-pair HPLC/ C18 column (100A, 150 mm × 4.60 mm)/gradient elution of phosphate buffer at pH3.0 (phase A) and methanol (phase B)	Extraction with borate buffer (for vitamins B2, B3amide, B5, B6, B9) and with 0.1 M sulphuric acid for vitamins B1 and B3acid	[208]
FoodproductMarmite	B1, B2,B6	Cyclic voltammetry in conjunction with screen printed carbon electrodes	Extraction with 0.2M trisodium phosphate buffer/ warmed to 30°C for 10 min, dilution of the supernatant	[197]

		(spces)	with 0.1M phosphate buffer (pH11.0) in voltametric cell with 0.1 M sodium chloride	
Cerebronorm powderedtablets	B2, B3	Ion-pair HPLC-UV /prontosil-120-5-C18 AQ column (75 mm ×2mm, 5 µm)/(A)0.4% KH2PO4 solution:0.185% sodium heptyl sulfonate solution:0.005% solution of triethylamine:0.25% acetic acid 1:1:1:1, (B) acetonitrile:water 1:1/ gradient elution	Dilution of the sample with 20 ml of water on heating to 40°C in a 50.0-ml volumetric flask /agitation for 5 min/dilution up to 50 mL with water/filtration through a “white tape” paper filter	[209]
Oral powder for veterinary consumption	B1, B2, B3 (nicotinamide), B6,C	RP-HPLC-PDA/ Zorbax XDB C18 column (250 mm x 4.6 mm, 5µm)/(A) 1.86 g/L 1-hexanesulfonic acid-sodium salt 1,5% acetic acid, methanol:mobile phase 90 (A):10 (B) v/v/ gradient elution	Dilution of the sample with 50 mL of distilled water into the 100mL volumetric flask /sonication for 2min/dilution up to the mark/dilution of 3.0 mL of the solution into 25 mL volumetric flask to the volume with mobile phase A/filtration through 0.45 µm RC filter	[210]
Pediatricsyrup	B1, B2, B3 (nicotinamide), B6, B12, C	UPLC-ESI-MS/MS / ACQUITY UPLC BEH Shield C8 column (2.1 mm x 50 mm, 1.7 µm)/ mobile phase: a mixture of 10 mM ammonium formate in 0.2% formic acid pH2.8 with methanol by gradient elution	Dilution of the sample with 50mL water into 100mL volumetric flask /sonication for 5 minutes/dilution up to mark/dilution of the solution with 10 mM ammonium formate in 0.2% formic acid at pH2.8/filtration with 0.2µm membrane filter	[211]
Cornsteepliquor	B1, B2, B3, B5, B6, B7, B9, B12	RP-HPLC-DAD/ Acclaim PA column (4.6 mm × 150 mm, 3 µm) and Acclaim PA guard column (4.6 × 10 mm, 5 µm)/	2 LLE and 4 enzymatic extractions were evaluated	[212]

		(A) 25 mmol/L KH2PO4 buffer, pH3.5, (B) acetonitrile/gradient elution		
Wheatflourproducts	B1, B2, B3, B5, B6,	LC-ESI-MS/MS / Nucleodur Polar tech column (Macherey-Nagel, EC 150/2.5 µm)/ (A) acidified water and(B) acidified MeOH(with 0,1% formic acid)	Extraction with sodium acetate (pH4.5)+0.5M glyoxylic acid solution+1%L-glutathione reduced solution+1% ethylene-diamine-tetraacetic acid solution+2% iron(II) sulfateheptahydrate solution/ addition of mixture of enzyme solution in sodium acetate (pH4.5) containing 2.5 mg of phosphatase, 12.5 mg of papain, 62.5 mg of taka-diastase, and 2.5 mg of β-glucosidase and incubation at 37 °C for 14 h	[213]
Infant/Adult Nutritional Formula and Breakfast Cereal	B1, B2, B3, B5, B6	LC-IDMS and LC-IDMS/MS / Cadenza CD-C18 column (250mm × 4.6 mm, 3 µm)/ (A)20M ammonium formate in water adjusted to pH4.0 with formic acid, (B) methanol	Extraction with 1 % acetic acid in water in ultrasonic bath for 30-120 min without heating/centrifugation/filtration of supernatant	[214]
Multivitamin preparations (effervescent tablets, tablets, capsule, caplets and ampules) and three liquid multivitamin drinks based on orange juice	B1,B3,B5,B6,B9, C, E,A	^{1}H NMR spectrometry	One extraction with 1 mL of thymol solution (1 mg/mL in MeOD)/centrifugation/two more extractions with pure MeOD/centrifugation of the liquid phases	[215]

Beverages and dietarysupplements	B1, B2, B3, B5, B6, B7, B9, B12, C	LC-MS/MS / Scherzo SM-C18 column (150 mm × 2.0 mm, 3 µm)/ (A) 5 mM ammonium formate 0.05% (v/v)formic acid and (B) acetonitrile0.3% (v/v) formic acid	Ultrasonically degassing for 10 min/dilution with water (beverages), extraction with water:acetonitrile (95:5 v/v) and acetic acid 1% (v/v) in a water bath at 65ºC for 10 min/centrifugation/further dilution of supernatant with water	[17]
Czechcheeses	B3, B5,B6	RP-HPLC-DAD/ Supelcosil LC 8 column (150mm x 4.6 mm; 5 µm)/ (A) 90% phosphate buffer (0.1M potassium dihydrogen phosphate, pH7.0) and (B) 10% methanol in isocratic elution.	Mix with 1% clara-diastase and 0.5 mL of fromaza solution/adjustment to pH 6.0 with 2M NaOH/water bath at 25^{o}C for 1h/oven at 30^{o}C for 20 h/ in 250 mL volumetric flasks, 1 mL of 80% trichloroacetic acid (TCA) and 2 mL of Carrez I and Carrez II + mixture + deionised water up to the mark/filtration through paper filter (Filtrapak, No.390) and 0.45 µm nylon membrane	[216]
Multivitamintablets	B1, B2, B3, B9, B12, C, A, D3, E, K1	RP-HPLC/ Phenomenex Gemini C18 110A column (150mm × 4.6 mm, 3.0)/ (A)0.01% TFA, pH4.0 and (B) 100% methanol/gradient elution	Various extraction solutions containing 0.01% TFA and methanol in different ratios (100:0, 80:20, 50:50, 20:80, and 0:100)/centrifugation/filtration of the supernatant	[217]
Orange juice, strawberry milkshake and malt	B1, B2,B3, B6, C	Multisyringe Chromatography (MSC)-DAD/ chromolithtm Flash RP-18e column (50mm x 4.6mm) / (A) 5mM sodium 1-hexanesulfonate, pH7.0 and (B) 5mM sodium 1-hexanesulfonate: methanol, 80:20 (v//v)/isocratic elution	Extraction with 15mL of aqueous mixture of acetic acid 2.4% (v/v) and 1% (w/v) of m-phosphoric acid/overnight storage at 4^{o}C/ultrasonic bath for 15 min/ filtration through 0.45mm Nylon membranes	[218]

Ready-to-eat green leafy vegetable products including green lettuce, ruby red lettuce, watercress, swiss chard, lamb's lettuce, spearmint, spinach, wild rocket, pea leaves, mizuna, garden cress and red mustard	B1,B2,B3,B5,B6, B9, C, E, provitamin A	HPLC–DAD–MS/MS/ACE-100 C18 column (100 mm ×2.1 mm, 3µm) / (A) 10mM ammonium acetate solution, pH4.5, (B) methanol 0.1% acetic acid and (C) methanol 0.3% acetic acid/gradient elution	Extraction with 16 mL of 10mM ammonium acetate:methanol 50:50(v/v) containing 0.1% BHT+40µL of 1 mg/mL hippuric acid and 40µL of 1mg/mL trans-β-Apo-8′-carotenal/shaking and ultrasound bath with ice for 15min/centrifugation/filtration of the supernatant through a 0.45µm nylon filter	[219]
Human urine and Pharmaceutical formulations	B1, B2, B3, B6, B12, B9, C	RP-HPLC-UV-Vis/ Alltima C18 column (250 mm × 4.6 mm, 5 µm)/ (A) 15 mM ammonium formate buffer containing 0.1% triethylamine, adjusted to pH4.0 with formic acid and (B) acetonitrile/gradient elution	Extraction with formic acid in a ratio (1 + 2, v/v)/sonication/centrifugation/ 5 mL of the supernatant were diluted with the diluent in a ratio of 1 + 10 (v/v)/filtration of the supernatant through a 0.22 µm PVDF syringe filter **(for human urine)** in 50mL volumetric flask/ extraction with 10 mL 1.0% ammonium hydroxide solution/ sonication in the dark for 15 min/ addition of 20mL 5% acetonitrile in 0.01 M HCl solution and 10mL 0.1% m-phosphoric acid solution/ heating for 5 min at 60–70°C in a water bath/dilution up to volume with the diluent/centrifugation of the supernatant and filtration through a 0.22 µm PVDF syringe filter **(for pharmaceutical formulations)**	[220]

Dietary supplements and infant powder milk	B1, B3 (nicotinic acid), B5, B6,B7, B9, C	on–line HPLC coupled to a corona-charged aerosol detector/Lichrosorb RP-C18 column (5 µm, 250 mm×4.6 mm)/0.05M ammonium acetate: methanol 90:10 (v/v)/isocratic elution	Dilution in ultrapure water (25mL)/ultrasound treatment for 1 min **(dietary supplements)** dilution of the sample in 25mL of heated (40-45ºC) ultrapure water/addition of 10mL 2.5M acetic acid/centrifugation **(for infant powder milk)**	[221]
Multivitamin/Multi element Tablets and Infant/Adult Nutritional Formula	B3, B1, B2, B5, B6	LC-ESI-MS/ Cadenza CD-C18 column (250 mm×4.6 mm, 3 µm)/ (A) 20 mM ammonium formate, pH4.0 and (B) methanol/gradient elution	Extraction with 24mL 1% acetic acid in water/sonication (no heat) for 30min/centrifugation/filtration of 5mL supernatant through a 0.45 µm nylon filter **(for Multivitamin/Multielement Tablets)**. Extraction with 29mL 1% acetic acid in water/sonication (no heat) for 30min/addition of 50 µL acetonitrile/ freezing (-20°C) overnight/centrifugation/filtration of 5mL through a 0.45 µm RC filter **(for Infant/Adult Nutritional Formula)**	[19]
Honey	B2, B3 (nicotinic acid), B5, B9, C	RP-HPLC-UV-Vis/Alltima C18 column (250 mm×4.6 mm, 5µm) with a guard cartridge/ (A) aq. solution of trifluoroacetic acid (0.025%, v/v) and (B) acetonitrile/gradient elution	Addition of 12.5 mL of phosphate buffer 1M (pH5.5) in aqueous solution of the sample in a 25 mL volumetric flask/filtration of the solution through a PVDF (13 mm and 0.45 µm)	[222]

Tears andblood serum of infants and parents	B1, B2, B3 (nicotinamide), B5, B9	LC-ESI-MS/MS / C18 silicacolumn(150 mmx2.1mm, 3μm) with a guard cartridge/(A) 0.1% FA in water and (B) ACN/gradient elution	The tear strip was submerged in 0.1% FA and vortexed for 15 s/centrifugation/incubation at 4^{o}C for 10min/injection of the supernatant **(for tears)** 100mL plasma was mixed with 400mL of MeOH/ACN/Acetone,1:1:1 (v/v/v)/ incubation at 4^{o}C for10 min/centrifugation/ drying under nitrogen/reconstitution in 100mL of 0.1% FA in water **(for blood serum)**	[53]
Tomato samples and pharmaceutical formulations	B1, B2, B3, B6, B9, C, A, E, K1	RP-HPLC-UV/C18 Kinetex column(100mm×4.6mm,2.6μm), coupled with a C18 guard cartridge (4mm×3.0mm)/ (A)0.1% FA in water, (B) acetonitrile and(C) acetonitrile/methanol/hexane (90/8/2, v/v/v) containing 0.1% acetic acid/gradient elution	Extraction with 15mL of 0.1% (w/v) meta-phosphoric acid/sonication in the dark for 18min/centrifugation/filtration of the supernatant through 0.2μm RC filters/addition of 2% meta-phosphoric acid up to 15mL/addition of 2.5mL of a reducing solution containing 1% w/v cysteine to 5mL of each filtrate/addition of 180μL of a diluted 1:5 v/v ammonia solution to get a pH around 7.0/decrease of pH to 2.5–2.8 with 700μL of 20% (w/v) meta-phosphoric acid	[223]

Energy and sportdrinks	B1, B2, B3, B5, B6, B12, C	Micellar electrokinetic capillary chromatography with PDA and uncoatedfused-silica capillariesof 58.5 cm length (50 cm effective length) x 75mm (375mm o.d.)	Energy and sport drinks were filtered directly through 0.45mm nylon filter and injected in the CE system. For carbonated samples, a previous sonication for 20 min to remove dissolved gases was performed. Fruit nectars with fortified vitamins were centrifuged at 10,000 rpm for 10 min, being the supernatant filtered and subsequently injected. Samples containing vitamin C were diluted 1:1 (v/v) with ice-cold metaphosphoric acid (up to a final concentration of 2%), filtered and injected.	[224]

CHAPTER 3

RESEARCH OBJECTIVE AND SCOPE

3.1 The analytical problem

The determination of vitamins in foods is often a challenging task due to their instability. Aging, storage conditions, and processing of foods cause vitamin loss, which varies widely depending on the type of food and several specific parameters (e.g., temperature, oxygen, light, moisture, pH) [225]. Vitamins are often present in foodstuff at trace levels, so their determination demandsa technique capable of detecting them at very low concentrations with satisfactory accuracy. Furthermore, the fact that foods are complex matrices makes even more difficult their extraction and their isolation and as a result thedevelopment of a method with the appropriate sample preparation before the analysis. Finally, as far as concernedthe simultaneous determination of vitamins, their different structures and chemical properties have to be taken into consideration [205].

3.2 Scope

The aim of this study was to develop a fast, reliable and cost-effective and accurate method for the simultaneous determination of water-soluble vitamins in cereal matrices with LC-MS/MS. After a brief literature review, LC-MS/MS was considered the best technique for the simultaneous determination of vitamins, providing the best selectivity and sensitivity.

The sample preparation was optimized by testing various extraction solvents and the extractions' conditions like the extraction time, the use of sonication and shaker.

Finally, the method was validated in agreement with the guidelines of CommissionDecision 2002/657/EC and satisfactory method performance characteristicswere achieved. The method was applied to the analysis of commercial samples and a proficiency test in order to ensure the accuracy of this method.

CHAPTER 4

INSTRUMENTATION, LABORATORY EQUIPMENT AND REAGENTS

4.1 Instrumentation of LC-MS/MS system

A Thermo UHPLC Accela system was connected to a Thermo Scientific Quantum (TSQ) Access Triple Quadrupole Instrument (Thermo, San Jose, CA, USA) equipped with ESI source, a UHPLC pump (Thermo Accela) and an Accela autosampler (Figure 6). The triple quadrupole mass spectrometer was operated in multiple reaction monitoring (MRM) mode in positive and negative ionization mode.

Instrument control and data acquisition were carried out by using the Xcalibur software, Version 2.3, from Thermo.

Figure 6: Thermo Scientific TSQ Quantum Access Triple Quadrupole Instrument [226]

4.2 Laboratory equipment

In the laboratory equipment used were included column Atlantis T3 C18 (100 mm x 2.1 mm, 3 mm, Waters), mobile phase solvent filtration apparatus (Millipore, XX15.04705), calibrated analytical balance with four decimal digits (Santorius-Basic), ultra-pure water apparatus 18.2MΩ/cm (Millipore Direct-Q UV), Optoelectronically controlled small shaker (VXR basic Vibrax®),ultrasonic bath

(Metason 60 Stuers), a Vortex spinner apparatus (Velp Scientifica), a centrifugation apparatus (Neya 16R) and a pH meter (HQ30d, HACH). They were also used10, 50, 100 and 250 mL volumetric flasks, 100and 250 mL beakers, 15 and 50 mL centrifuge tubes, 10 and 25 mL volumetric cylinders, calibrated 100μL, 200μL, 1mL and 5 mL pipettes, and plastic pasteur pipettes.

4.3 Chemicals and Reagents

All vitamin standards and hippuric acid were of high purity grade (≥97%), as the Table 4 shows.

Table 4: Purity and supplier of vitamin standards

Vitamin	%Purity	Supplier
Thiaminehydrochloride	≥99%	Sigma-Aldrich
Rivoflavin	98%	Alfa Aesar
Nicotinicacid	99%	Alfa Aesar
Nicotinamide (Niacinamide)	100%	Supelco
D-Pantothenic acid hemicalcium salt	≥99.0%	Sigma-Aldrich
Pyridoxinehydrochloride	99%	Alfa Aesar
Biotin	≥99%	Sigma-Aldrich
Folicacid	≥97%	Sigma-Aldrich
Vitamin B12	≥98%	Sigma-Aldrich
L-Ascorbicacid	99.2%	Supelco
Hippuricacid	98%	Sigma-Aldrich

Methanol LC–MS grade was purchased from Merck (Darmstadt, Germany), while formic acid ~98% from Honeywell (Charlotte, North Carolina, USA) and ammonium formate from Fluka (Buchs, Switzerland). Chloroform (puriss. p.a., reag. ISO, reag. Ph. Eur., 99.0-99.4% (GC)) was purchased from Sigma–Aldrich (Steinheim, Germany) and distilled water was provided by a MilliQ purification apparatus (Millipore DirectQ UV, Bedford, MA, USA). As far as ammonium acetate, 2,6-Di-tert-butyl-4-methylphenol (BHT) and Trichloroacetic acidare concerned, they were purchased Merck, Alfa Aesar and Riedel-de Haën (Seelze, Germany), respectively. RC Chromafil (regenerated cellulose) syringe filters (15 mm diameter, 0.2 μm pore size) were provided from Macherey-Nagel (Duren, Germany).

4.3.1 Preparation of standard solutions

Stock standard solution of 1000 mg/L was prepared monthly by weighted 10 mg of each analyte in a 10 mL volumetric flask and diluted to volume with distilled water.For vitamins B2, B3 acid, B7, B9, and hippuric acid, a few drops of sodium hydroxide solution (1M) were added to facilitate the dissolution.As far as ascorbic acid, it was diluted in 2% meta-phosphoric acid (MPA) in order to avoid its oxidation.

Working standard solutions of 100 mg/L were prepared daily in distilled water from the stock solutions, in order to construct the calibration curves and the standard addition curves of the analytes. The working standard solution of200 mg/L of hippuric acid, used as internal standard, was also prepared daily in distilled water.

Solid standards and stock solutions were kept at 4^{o}C. To prevent photo degradation, the stock solutions were stored to amber glass vials for up to one month.

4.3.2 Samples

Fortified breakfast cereals were used during the optimization experimentsof this method andthey were all commercially obtained from the Greek food markets. Since blank samples were not available, a surplus of cereal proficiency test (PT) from BIPEA (6-7020 - Cereals with vitamins Caloric value Minerals Vitamins) was used for the method validation.

Commercial breakfast cereals were homogenized and stored at room temperature, while the surplus of BIPEA PT at freezer (-20^{o}C).

For the evaluation of the different extraction procedures, samples of fortified breakfast cereal were spiked at 3 fortification levels as presented in Table 5. Afterwards, there was a waiting period of 30 min for equilibration before starting the extraction step.

Hippuric acid was selected to be used as Internal Standard (IS) [227]. A constant concentration of it was added at each sample in the middle of the sample preparation to achieve a final concentration of 1 mg/L.

Spiked samples were prepared by adding the proper amount of a working solution containing all the analytes at the suitable concentrations, to each portion of the weighed samples.

Table 5: Vitamins' concentrations according to cereal's label and the levels of concentration that were spiked

Vitamin	Label's concentration (mg/kg)	Spiked concentration (mg/kg)		
		50%	100%	200%
B1	18.0	10.0	20.0	40.0
B2	23.0	12.5	25.0	50.0
B3 (acid&amide)	134	67.5	135	270
B5	-	10.0	20.0	40.0
B6	12.0	7.50	15.0	30.0
B7	-	0.25	0.50	1.00
B9	3.34	1.75	3.50	7.00
C	-	500.0	1000	2000

CHAPTER 5

METHOD DEVELOPMENT FOR THE DETERMINATION OF WATER-SOLUBLE VITAMINS IN CEREAL BY LC-MS/MS

5.1 Optimization study of LC-ESI-MS/MS analysis

5.1.1 Ionization study

As the first step of the method development, the selection of the precursor and product ions was carried out. Direct infusion of individual vitamins at concentration of 1-5mg/L in distilled water:MeOH (80:20, v/v)was achieved in positive and negative ionization mode. The mass spectra for all analytes were obtained along with analyte dependent parameters, such as collision energy and tube lens, which were optimized and calculated automatically. For each compound, the MRM transition with the highest intensity was used for quantification (quantifier), while the other transition was used for confirmation (qualifier). A quantitative data processing method was established using the most abundant SRM (Selected Reaction Monitoring) transition for each residue.

Electrospray parameters, such as sheath gas pressure, auxiliary gas pressure, spray voltage and capillary temperature, were studied. The optimization was performed using flow injection analysis (FIA) with the carrier solution being the analysis' mobile phase in different proportions of aqueous/organic solvent. MS parameters were optimized in positive and negative ionizationmode with variation of a single setting at a time and evaluation of the target compounds' sensitivity. Significant compromises had to be made in order to simultaneously determine all analytes with different optimum values of ESI parameters.

5.1.2 Optimization of mobile phase

Several different combinations of mobile phases were tested, which at any occasion, consisted of an aqueous buffer and an organic solvent, at the same ratio (90:10). The buffers tested were either ammonium acetate or ammonium

formate at different concentrations (1mM, 5mM or 10mM) at different pH values (pH 7.0, pH 4.5 or pH 3.5) and acidified in three different concentrations with formic acid (0.1%, 0.05% or 0.01%). As organic phase, methanol was used, acidified or not with 0.1% formic acid. InTable6 are presented all the combinations of mobile phases tested.

Table6: Mobile phases tested

Test	Aqueous phase / Organic phase
A	10mM ammonium acetate pH 4.5
	10mM ammonium acetate pH 3.5
	MeOH
B	10mM ammonium acetate pH 4.5
	MeOH
C	10mM ammonium acetate pH 3.5
	MeOH
D	10mM ammonium acetate pH 7.0
	MeOH
E	10mM ammonium formate 0.1% FA
	10mM ammonium formate in MeOH 0.1% FA
F	10mM ammonium formate 0.1% FA
	MeOH
G	10mM ammonium formate pH 3.5
	MeOH
H	10mM ammonium formate pH 4.5
	MeOH
I	10mM ammonium formate 0.1% FA
	MeOH - 0.1% f.a.
J	5mM ammonium formate 0.1% FA
	MeOH
K	10mM ammonium formate 0.05% FA
	MeOH
L	10mM ammonium formate 0.01% FA
	MeOH

The optimum one was chosen in terms of highest peak area and signal-to-noise ratio for the majority of the target compounds. The optimal mobile phase was 10mM ammonium formate with 0.05% formic acid:MeOH (90:10).

5.1.3 Optimization of elution program

Due to the different chemical structure of the analytes, three different gradient programs were tested. The gradient programs were applied not only to a

standard solution but also to a sample in order to verify the best chromatographic separation and peak shape. The gradient elution programs are presented in Table 7 and the optimal gradient program was the first one (A). The mobile phase used was 10mM ammonium formate with 0.05% formic acid:MeOH(90:10), as described above in Section 5.1.2.The column temperature was set at 30 °C and the injection volume of the extract was set at 10 μL.

Table7: Gradient programs tested

Gradient program A				Gradient program B				Gradient program C			
Time (min)	**%A**	**%B**	**Flow (μL/min)**	**Time (min)**	**%A**	**%B**	**Flow (μL/min)**	**Time (min)**	**%A**	**%B**	**Flow (μL/min)**
0.00	10	90	200	0.00	50	50	200	0.00	10	90	200
4.10	10	90	200	2.10	50	50	200	1.10	10	90	200
6.00	50	50	200	6.00	50	50	200	2.00	50	50	200
10.00	50	50	200	10.00	50	50	200	10.00	50	50	200
10.10	100	0	200	10.10	100	0	200	10.10	100	0	200
12.00	100	0	200	12.00	100	0	200	12.00	100	0	200
12.10	10	90	200	12.10	50	50	200	12.10	10	90	200
16.00	10	90	200	16.00	50	50	200	16.00	10	90	200
A: MeOH, B: 10mM ammonium formate - 0.05% formic acid											

5.2 Experimental procedure

A schematic diagram of the sample preparation (spiking, extraction and clean-up process) is presented in Figure 7.

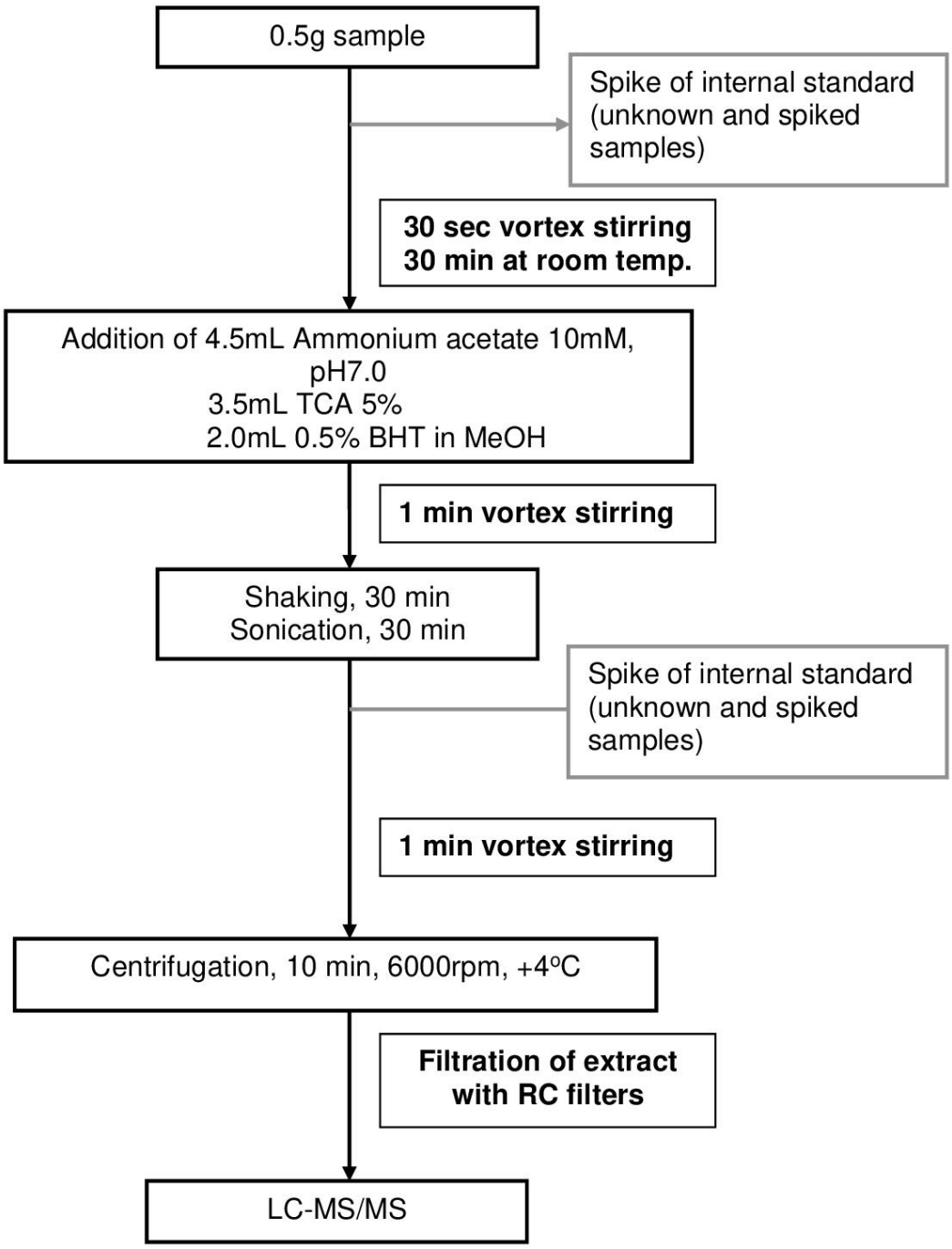


Figure 7: Schematic diagram of the final experimental procedure

5.3 Optimization of sample preparation

5.3.1 Mass of sample

The choice of the samples' weight constitutes an important step in the experimental procedure as it has to be adequate in order to detect the analytes of interest.

In case of solid samples, powders and suspensions, the mass of sample is more crucial parameter due to the inhomogeneous distribution of the analytes. In this study, 1g and 0.5g of sample were examined to test the homogeneity of the sample and the matrix effect.

5.3.2 Selection of buffer

The first of the solvents that are added to the sample, is an aqueous buffer with concentration of 10mM. In this part of the study, it was examined whether ammonium acetate or ammonium formate is the most efficient salt for the buffer. The concentration of the tested buffers was the same, 10mM.

Ammonium acetate had been selected as it was often used in the literature [206], [219], whereas ammonium formate because it was compatible with the mobile phase. However, ammonium acetate was proved to be more efficient for the extraction of vitamins.

5.3.3 pH of buffer

After the selection of ammonium acetate and TCA as extraction solvents, it was tested the pH of the buffer due to the fact that the water-soluble vitamins degrade under different pH values. So, it is important to be taken into consideration the pH of the buffer in combination with the addition of TCA. Thus, they were selected to be studied three different pH values of the ammonium acetate buffer, pH 4.5, 3.5 and 7.0.

5.3.4 Addition of acidic agent

According to literature [216]–[218], [221], the water-soluble vitamins were extracted with acidic solutions. Thus, six different acidic solutions with the same pH value were studied, so as to compare the efficiency of the acidic agents. The aqueous solutions that were tested were Hydrochloric acid (HCl) 0.1M, Perchloric acid 0.1M, Acetic acid 5M, Trichloroacetic acid (TCA) 9.8% w/v, Trifluoroacetic acid (TFA) 9.8% w/v, and meta-Phosphoric acid (MPA) 2% w/v and TCA was selected as the most appropriate acidic agent

5.3.5 Concentration of TCA

For the extraction of vitamins, TCA was considered the most efficient acid for the samples' preparation. In this section, it was optimized the acid's concentration and were examined two concentrations; 9.8% w/v and 5% w/v. The optimal concentration of the TCA was proved to be 5% w/v.

5.3.6 Concentration of BHT in MeOH

Finally, it was examined the concentration of BHT in MeOH. On one hand, BHT prevents the oxidation of the vitamins during the experimental procedure, and on the other hand MeOH helps the precipitation of proteins in cereals facilitating the sample's cleanup. Thus, three different concentrations of BHT were studied;1% w/v, 0.5% w/v, and 0.1% w/v.

5.3.7 Proportion of solvents

In this section, it was studied the ideal proportion of the three solvents used in order to achieve the most efficient extraction of the vitamins from the cereals. In Table 8 are presented the proportions of ammonium acetate (10 mM, pH 7.0), TCA 5%, and 0.1%BHT in MeOH in mL.

Table 8: Proportions of solvents tested

Test	Ammonium acetate, 10mM, pH 7.0 (mL)	TCA 5% (mL)	0.1% BHT in MeOH (mL)
A	5	4	1
B	5	3	2
C	4.5	3.5	2
D	4	4	2
E	4	3	3
F	3	5	2
G	3	3	4

The optimal method was achieved by adding 4.5mL of buffer, 3.5 mL of TCA and 2mL of methanolic solution to 0.5g of the cereal sample.

5.4 Optimization of extraction method

5.4.1 Defatting solvent

As cereal is a complex matrix rich in lipids and proteins, it was necessary the removal of the lipids in order to achieve a further clean-up of the samples. An important step of the samples' preparation that follows the dilution of the raw sample is the extraction of the vitamins. The extraction assists the removal of lipids of cereals for the more effective isolation of the analytes. Among hexane, chloroform and heptane, chloroform was finally selected, since it was proved that this extraction medium succeeded purest extracts with higher concentrations of the vitamins.

5.4.2 Shaking-sonication sequence

To extract the WSVs from cereals, shaking and ultrasonic-assisted extraction were selected as extraction methods.The duration of each extraction was set to 30 min. The purpose of this section was to compare the effect of the sequence of the two extraction methods. So, we came up to the conclusion that the samples should firstly be extracted by shaking and then with the use of sonication.

5.4.3 Extraction time

Afterwards, it was studied the extraction time of shaking and sonication. Thus, 10, 15, 20, 25, and 30 min were tested as extraction time of shaking and sonication respectively keeping constant at 30 min the other parameter. So, we concluded that the best combination for the extraction of WSVs was achieved with 30 min shaking and 30 min sonication.

5.5 Method validation

The applicability of the was tested following the acceptedcriteria for analytical method validation, as indicated in the Commission Decision 2002/657/EC for quantitative methods [228]. Method detection limit (LOD), quantification limit (LOQ), linearity, accuracy and selectivity, were determined forallthe analytes of interest in cereals.

Linearity was assessed by constructing standard calibration curves and standard addition curves for each vitamin in seven different concentration levels and expressed as the determination of coefficient (R^2). Matrix-matched standards were also analyzed to investigate the matrix effect. Precision was evaluated through repeatability experiments and expressed as the relative standard deviation (%RSD). Accuracy was calculated through the recoveries of the spiked samples. The samples were spiked at three different fortification levels for each vitamin (Table 26) in six replicates. The LOD and LOQ of each method and for each analyte were determined by spiking the analytes at the low concentration level that can be detectable in the instrument in ten replicates. For the analytes with high concentration in the cereal sample, the evaluation of the LOD and LOQ was conducted by diluting the sample in 4 different levels and calculating the LOD theoretically.

An in-house validation protocol was carried out, taking into consideration the requirements outlined in Commission Decision 2002/657/EC and Eurachem Method Validation Guide [228], [229]. The developed method was validated in terms of selectivity, linearity, accuracy (recovery), precision (repeatability), limit of detection (LOD) and limit of quantification (LOQ). The validation experiments were carried out using the standard addition method by spiking cereal samples with the proper amounts of vitamin's standards.

5.5.1 Identification

Identification and confirmation of the analytes were carried out by retention times, identification points of each analyte as required by the EU validation criteria, and relative ion ratio of selected SRM transitions. For each compound, the SRM transition with the highest intensity was used for quantification (quantifier), while the other transition was used for confirmation (qualifier).

An analyte was considered as positively identified and confirmed in a sample whenthe criteria established in the EU Commission Decision 2002/657/EC were met:

1) the ratio of the relative retention time of the analyte to that of the same analyte in standard solution was within ± 2.5 % tolerance,

2) the presence of two SRMs for the analyte of interest, and

3) the signal intensity ratios of the two SRM transitions (quantifier and qualifier ions) with those obtained using fortified samples were within the tolerance defined [228].

5.5.2 Selectivity/ Specificity

The selectivity of the method was evaluated by the analysis of 10 control samples from the same matrix. The absence of any signal at the same elution time indicated the absence of chemical or matrix interferences that may give a false positive signal.

5.5.3 Linearity

The linearity was assessed by constructing standard calibration, standard addition and matrix-matched curve for each vitamin in seven different concentration levels (10%, 25%, 50%, 100%, 150%, 200%, and 300% of the assigned values) as shown in Table 9.This number of levels was chosen in order to achieve the optimal concentration range for each target analyte, considering the large differences in the concentrations and the sensitivity of the vitamins. Peak area and peak area ratio of the analyte/IS were used as the analytical response versus concentration in all cases. Calibration curves were obtained by least-squares linear regression analysis and acceptable linear regression R^2 values were obtained for all compounds over the concentration ranges. The concentrations in each calibration curve are presented at Tables 10 and 11.

Table 9: Concentration of vitamins in surplus of cereal PT

Vitamin	Assigned value (mg/kg)
B1	13.0
B2	13.6
B3 acid	-
B3 amide	179
B5	1.20
B6	14.5
B7	-

B9	2.18
C	-

Table 10: Concentrations of each vitamin (mg/L) for standard solution curve

	B1	B2	B3 acid	B3 amide	B5	B6	B7	B9	C
std1	0.0750	0.075	0.250	0.0900	0.100	0.0750	0.0100	0.0150	5.00
std 2	0.187	0.187	0.625	0.225	0.250	0.187	0.0250	0.0375	12.5
std 3	0.375	0.375	1.25	0.450	0.500	0.375	0.0500	0.0750	25.0
std 4	0.750	0.750	2.50	0.900	1.00	0.750	0.100	0.150	50.0
std 5	1.12	1.12	3.75	1.35	1.50	1.125	0.150	0.225	75.0
std 6	1.50	1.50	5.00	1.80	2.00	1.50	0.200	0.300	100
std 7	2.25	2.25	7.50	2.70	3.00	2.25	0.300	0.450	150

Table 11: Concentrations of each vitamin (mg/kg) for spiked sample and matrix-matched sample curve

	Fortification level	B1	B2	B3 acid	B3 amide	B5	B6	B7	B9	C
Spike1	10.0	1.50	1.50	5.00	18.0	2.00	1.50	0.200	0.300	100
Spike 2	25.0	3.75	3.75	12.5	45.0	5.00	3.75	0.500	0.750	250
Spike 3	50.0	7.50	7.50	25.0	90.0	10.0	7.50	1.00	1.50	500
Spike 4	100	15.0	15.0	50.0	180	20.0	15.0	2.00	3.00	1000
Spike 5	150	22.5	22.5	75.0	270	30.0	22.5	3.00	4.50	1500
Spike 6	200	30.0	30.0	100	360	40.0	30.0	4.00	6.00	2000
Spike 7	300	45.0	45.0	150	540	60.0	45.0	6.00	9.00	3000

5.5.4 Precision

The precision of this method was only demonstrated in term of repeatability (intraday precision). It was expressed as the %RSD values of set of 6 replicates under the same conditions at three different fortification levels (0.5, 1 and 2 times the assigned value).

5.5.5 Trueness

The trueness of the method was estimated through the evaluation of the recoveries. The recovery each analyte was examined in three different fortification levels in 6 replicates.

5.5.6 LODs & LOQs

LODs and LOQs of the method were estimated by analyzing ten samples. In case of vitamins B3 acid, B7 and C, ten replicates of spiked samples at low concentration were analyzed (1st fortification level of standard addition curve).

LODs were determined by 3.3 times the SD of the peak area of the vitamin divided to the slope of its standard addition calibration curve. LOQs were calculated multiplying 3 times the LODs. The instrumental LODs and LOQs were determined with the same way but from the standard calibration curves. The results were shown in Chapter 6.

For vitamins B1, B2, B3 amide, B5, B6 and B9, the estimation of LODs and LOQs was conducted by diluting the sample in four different levels, constructing a curve of the diluted samples and calculating from the equation the LODs.

5.5.7 Matrix effect

When complex samples, such as cereals are analyzed through LC–MS/MS, matrix effect is a parameter that has to be studied when ESI ionization was used.

Matrix effects derive from various physical and chemical processes and may be difficult or impossible to eliminate. Matrix effects can affect the ionization efficiency of the analytes, leading to suppression or enhancement of the signal depending on the analyte/matrix combination. Obviously, this affects the quantification, unless matrix effects are minimized or compensated. The best way to compensate the matrix effect is the use of isotope labelled internal standards (ILIS). However, these compounds are not always available and increase severely the cost of the analysis. The use of analogue ILIS is not always satisfactory. Therefore, matrix-matched calibration and standard addition method can be used alternatively.

To evaluate matrix effect, the slopes obtained from matrix-matched and standard calibration curves were compared. Matrix Effect (ME%) was calculated according to the follow equation:

$$\%ME = \left(\frac{Slope\ matrix - matched\ curve}{Slope\ standard\ curve} - 1\right) * 100$$

The signal enhanced when the %ME is positive, whereas it is suppressed when it is negative. A signal enhancement or suppression effect is considered acceptable if the %ME range from -20% to +20%.

5.5.8 Application to real samples

To evaluate the applicability of the proposed method, 2 cereal samples from market were analyzed. Three replicates of each sample were analyzed to compare the results with the labelled values. Thus, two different cereal samples with the same concentrations of vitamins were analysed:

1. Classic cereal
2. Cereal with red fruits

According to the European Commission guidance, there are tolerances for vitamins that ranged between +50% and -35% of the declared value including the uncertainty of the measurement [9].

CHAPTER 6

RESULTS AND DISCUSSION

6.1 Optimization study of LC-ESI-MS/MS analysis

6.1.1 Ionization study

The ultimate goal of this study was the development of an effective method for the simultaneous determination of water-soluble vitamins. Since vitamins' different physicochemical properties render their simultaneous determination quitedifficult, an extended investigation of their chromatographic behaviour was performed in order to increase their sensitivity and make their simultaneous chromatographic detection efficient.

Initially, experiments for the selection of the precursor and product ions for the 9 vitamins under full-scan MS mode's conditions, were carried out. Direct infusion of individual standards of each compound in distilled water: MeOH (80:20, v/v) was performed in positive ionization mode for the vitamins of B-complexand in negative ionization mode for vitamin C.

From previous studies, the mass spectra of the vitamins revealed $[M+H]^+$ ions as base peaks for vitamins of vitamins of B-complex except from vitamins B12 that was ionized as $[M+2H]^{2+}$. Vitamin C can be ionized either in positive or negative mode. However, the mass spectra obtained in negative ionization mode showed higher relative abundances of the target analytes than in positive ionization mode. In Figure 8 are presented the breakdown curves and the MS/MS spectrum of vitamin B3, B7, B9, B12 and C.

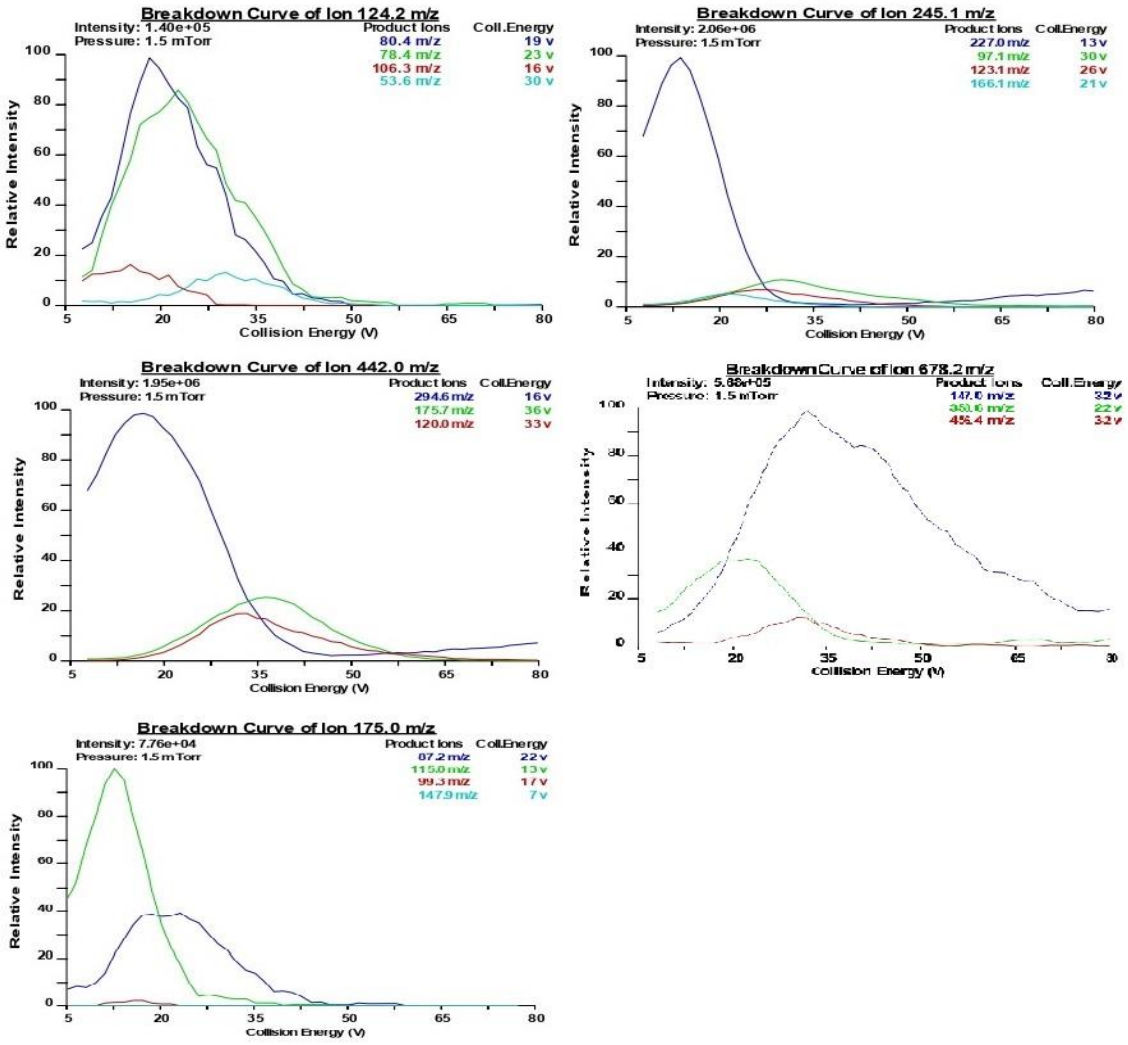


Figure 8: Breakdown curves for precursor ions of vitamins B3, B7, B9, B12 and C

In Table 12 are presented the precursor and the product ions as well as the Collision Energy (CE) of each vitamin.

Table 12: Product ions and ionization parameters of vitamins

Standard	ESI mode	Precursor Ion	Product Ions*	CE (eV)
Thiamine hydrochloride (B1)	(+)	265.10	**122.10**	10.0
			144.10	16.0
Riboflavin (B2)	(+)	377.00	**243.00**	23.0
			147.10	37.0
Nicotinic acid (B3)	(+)	124.10	**80.40**	19.0
			78.40	23.0
Nicotinamide (B3)	(+)	123.00	**80.30**	16.0
			78.30	24.0

D-pantothenic acid hemicalcium salt (B5)	(+)	220.10	**202.10**	11.0
			184.10	12.0
Pyridoxine hydrochloride (B6)	(+)	169.90	**152.10**	11.0
			134.10	19.0
Biotin (B7)	(+)	245.00	**227.00**	15.0
			97.00	33.0
Folic acid (B9)	(+)	442.00	**295.00**	13.0
			176.00	34.0
Vitamin B12	(+)	678.20	**147.00**	32.0
			358.60	22.0
Ascorbic acid (C)	(-)	174.90	**114.99**	14.0
			87.20	19.0
	(+)	177.00	**95.00**	11.0
			141.90	5.0
Internal Standard				
Hippuric acid	(+)	180.10	**105.20**	14.0
			78.10	21.0

***Quantifiers are in bold**

The optimal mass spectrometry settings are presented in Table 13.

Table 13: Mass spectrometric settings during the ionization study

MS Parameters	
Spray voltage (V)	4500
Sheath gas pressure (a.u.)	25
Auxiliary gas pressure (a.u.)	15
Capillary temperature (^{o}C)	270

6.1.2 Mobile phase optimization

As shown from the figures below, there are differences in the shape and the intensity of the peaks. The more acidified mobile phases, such as Test F, destroy the chromatographic image of some vitamins, such as niacin, while enhance the sensitivity of others. The selected mobile phase provides the optimal conditions for all the vitamins. It is finally selected Test K with 10 mM ammonium formate–0.05% formic acid:MeOH (90:10) as the optimum mobile phase.

Firstly, the first four tests are compared according to the shape and the intensity of niacin's peak in a standard solution of 27 mg/L, since it was the

vitamin which was most differentiated in these four mobile phases (A-D). As shown from the Figure below, the best chromatographic image was achieved with test D (10 mM ammonium acetate, pH 7) that improved not only the peak shape but also the intensity of the analyte.

As a second step, it was examined the efficiency of ammonium formate and its pH value. In Figures 10-12, it was presented characteristically a standard mix solution of tests D, K and L. From all the trials conducted, test K was finally selected, due to higher intensity and better shape of peaks.

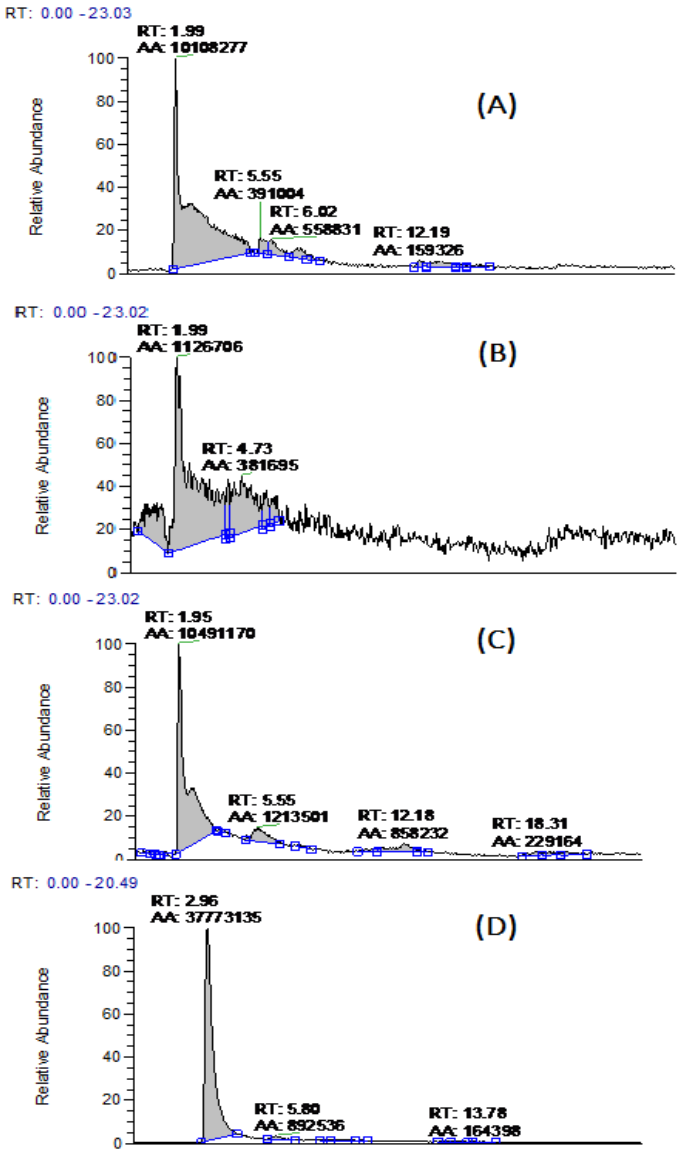


Figure 9: Chromatogram of nicotinic acid in different mobile phase's tests (A-D)

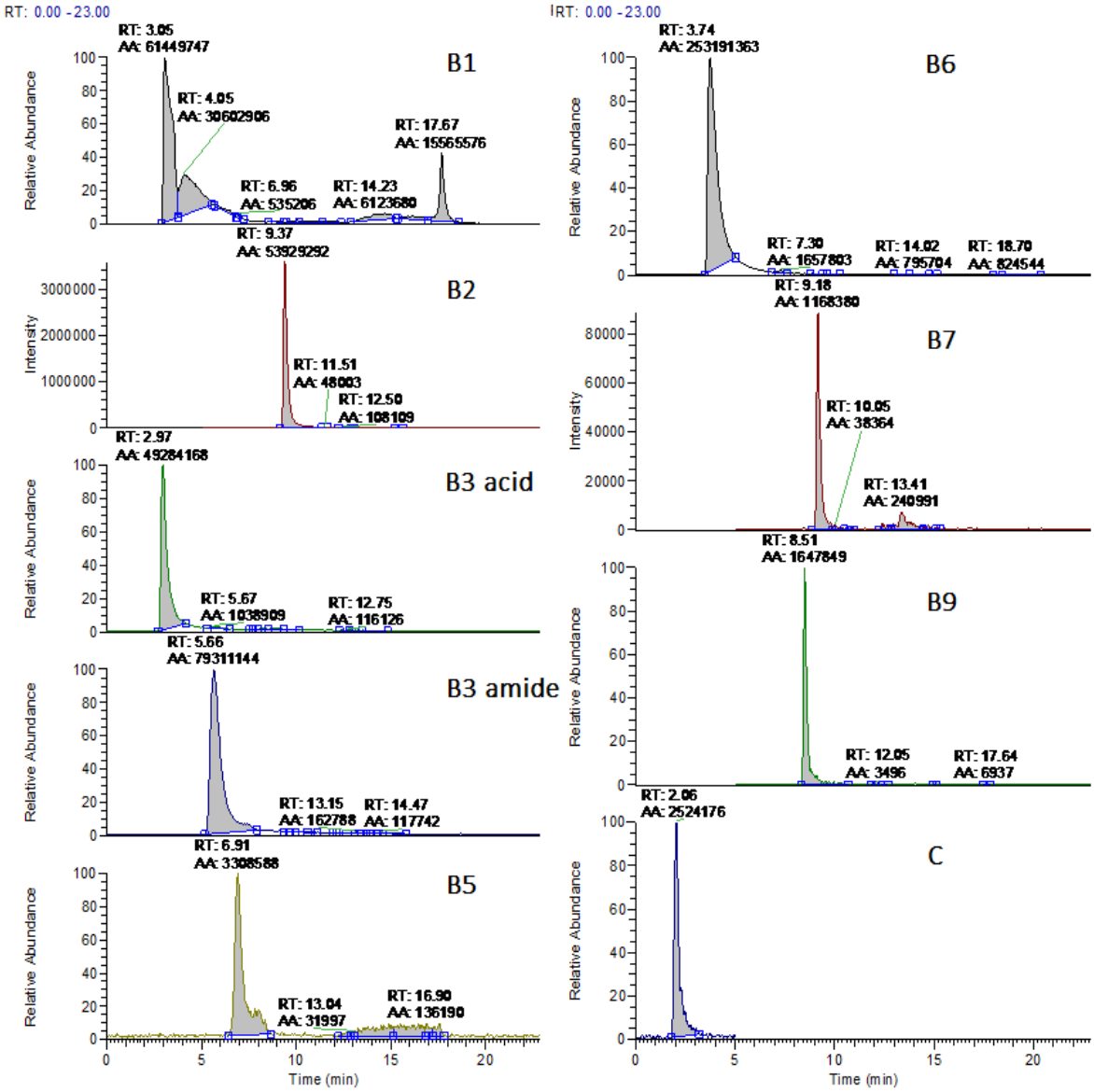


Figure 10: Chromatogram of vitamins in mobile phase's test D

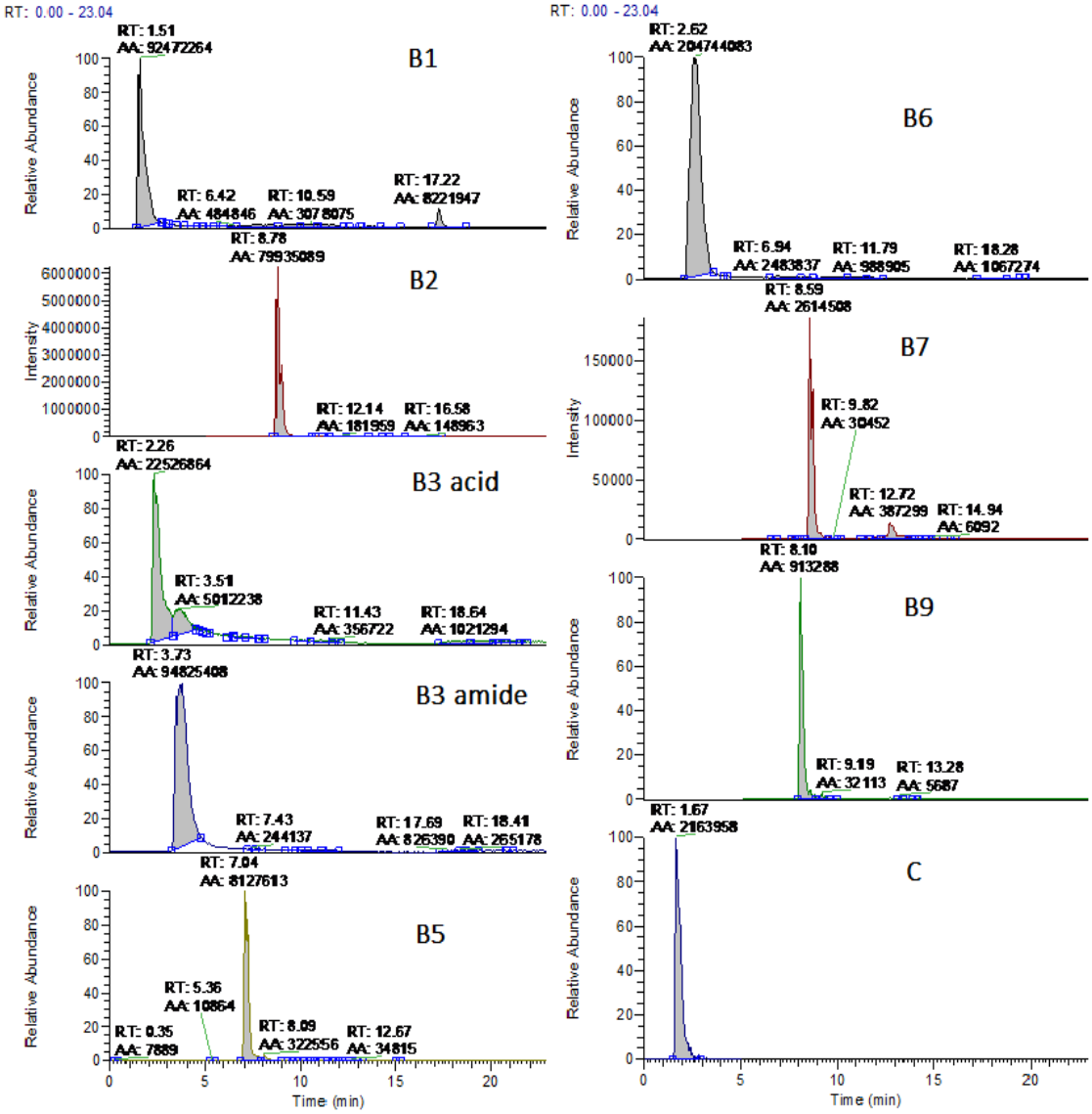


Figure 11: Chromatogram of vitamins in mobile phase's test K

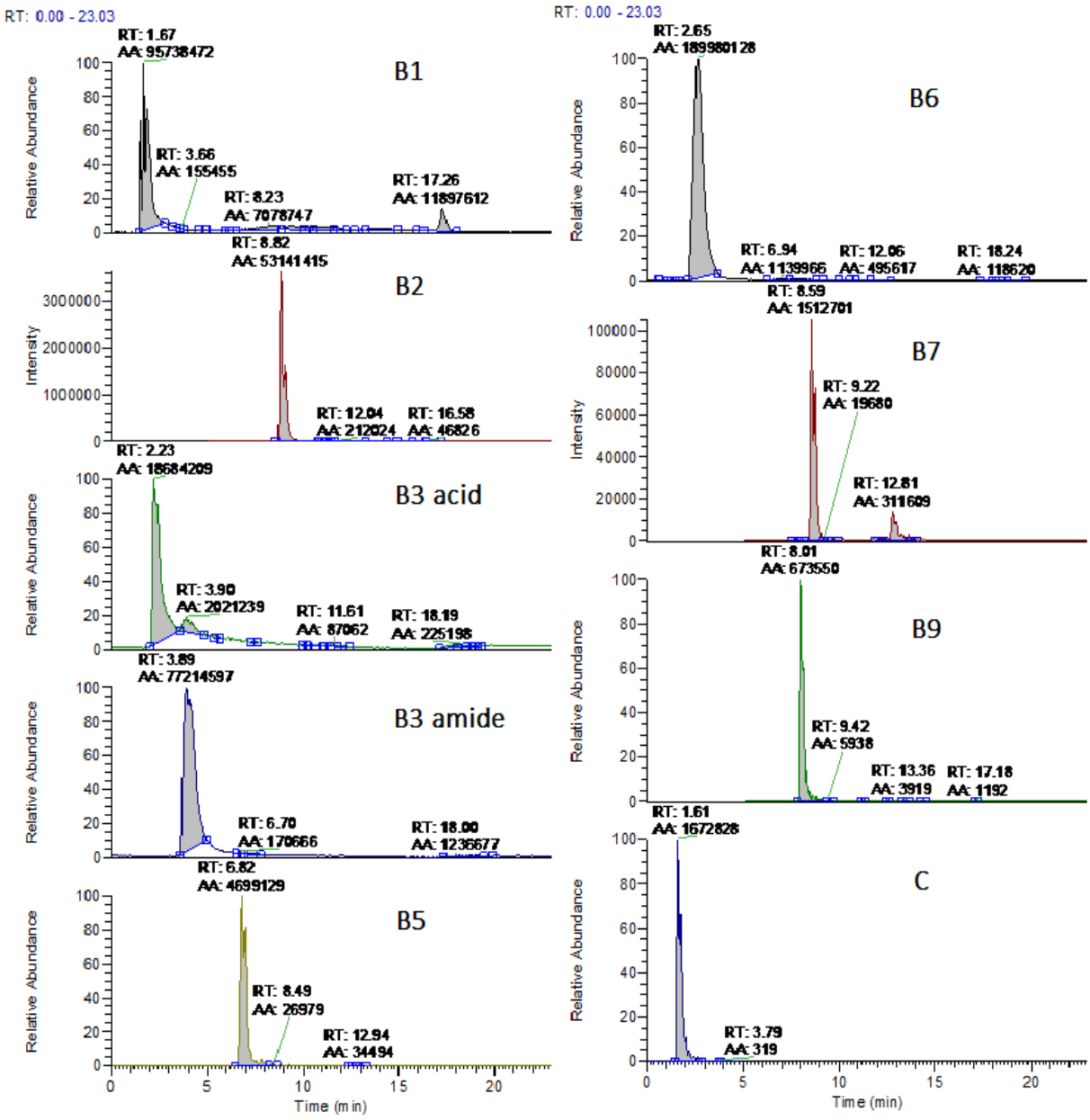


Figure 12: Chromatogram of vitamins in mobile phase's test L

6.1.3 Elution program optimization

Three gradient programs were tested. The first and the third oneare differentiated at the time that the percentages of aqueous (A) and organic (B) phase change from 90 (A): 10 (B) to 50 (A): 50 (50) in 1.10 and 4.10 min,whereas previously aqueous phase with the acidified buffer was prevailing. As far as concernedthe second test, it consists of equal percentages of aqueous and organic phase for 10 minand then organic phase

prevails for the next 2 min. These trials were conducted in order to improve the chromatographic image as well as the intensityof vitamins B1, B3, B6 and C that were eluted in the first four minutes of the chromatogram.

As shown in Figures 13 and 14, gradient program A seems to give more satisfying results.

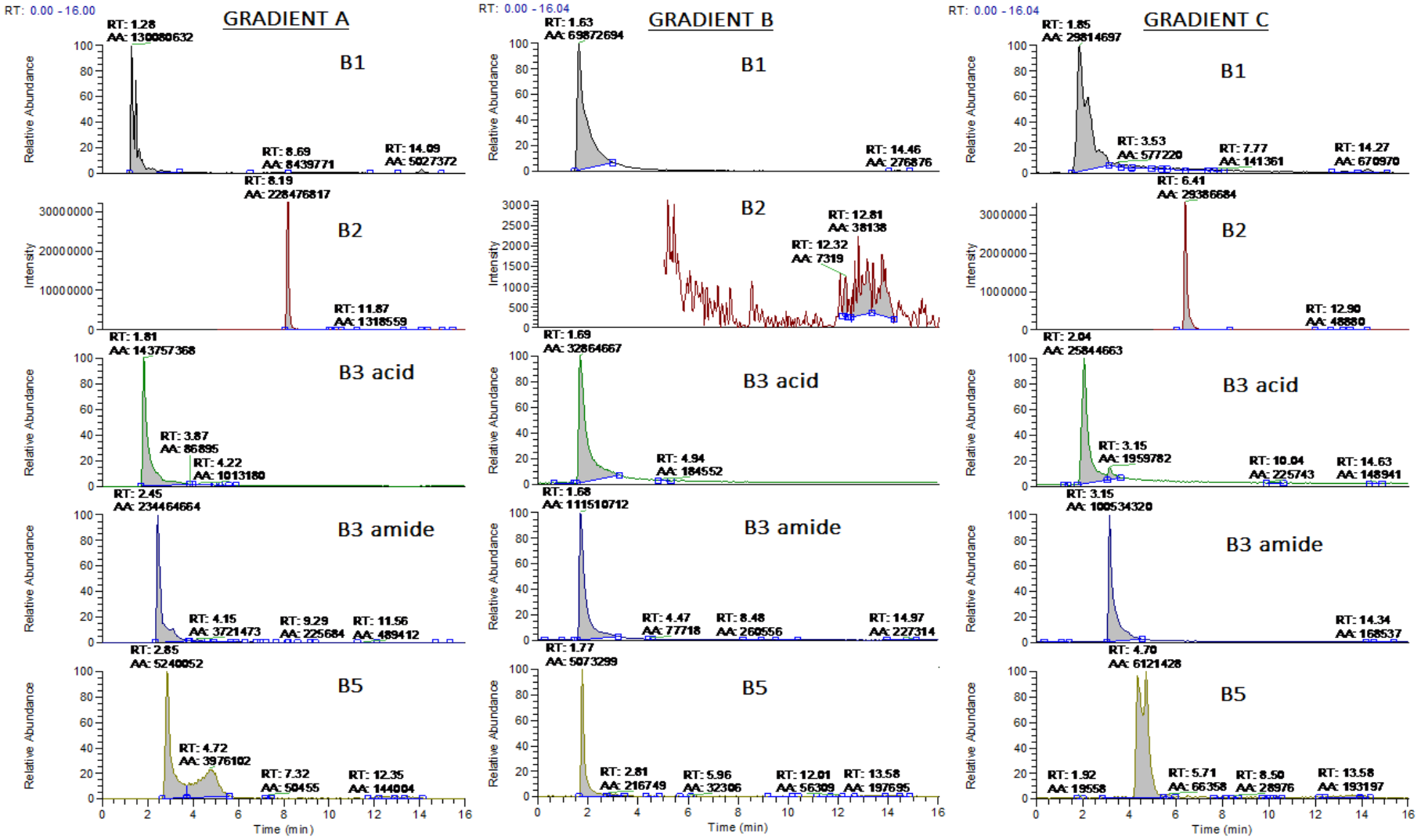


Figure 13: Chromatogram of vitamins B1, B2, B3, and B5 in the three gradient programs

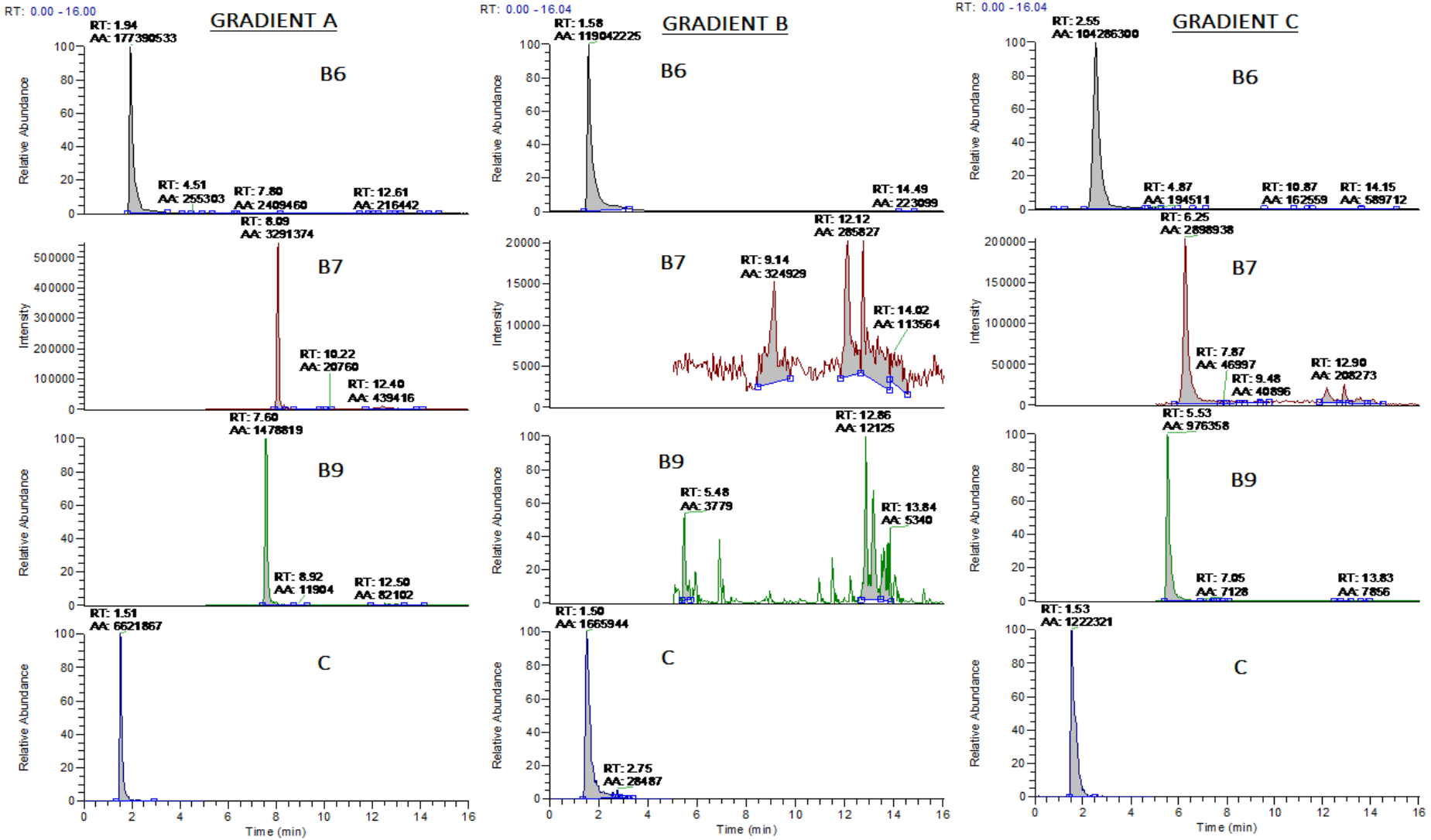


Figure 14: Chromatogram of vitamins B6, B7, B9, and C in the three gradient programs

6.2 Optimization of sample preparation

In this section the results of all the optimization tests as far as the sample preparation is concerned, are presented. The method is referred to all the water-soluble vitamins, except for B12, becauseit was proved through all the trials that the specific extraction procedure was not suitable for its detection due to its extremely low concentration in this matrix. For its isolation, it is required an extraction including an SPE step for the purification and the preconcentration of the analyte.

The fortification levels that have been chosen correspond to the percentages 50%, 100% and 200% of the labeled quantities of the vitamins in the cereal product that is examined. The concentrations of the vitamins, which do not exist in the product (B5, B7 and C), were selected according to literature and other cereal products from the market.

The quantification of spiked samples in the optimization experiments was conducted by the standard calibration curves. For vitamins B1, B2, and B6, it was used hippuric acid as internal standard as the results was significantly better. Thus, the optimization and the validation results of these vitamins were calculated with the ratio of area of the analyte to the area of hippuric.

6.2.1 Mass of sample

To examine the homogeneity of the sample and the matrix effect, two masses (1g and 0.5g) were tested. 0.5g was proved to give better results in comparison with those with 1g of sample, where the recoveries were significantly lower (Table 14) especially for vitamins B3 amide and C This may be occurred due to the high concentration of these vitamins that cannot be fully extracted from the matrix.In addition, the chromatographic image of vitamin B3 is better in 0.5 g because of the greater dilution of the analyte.

Table 14: Results of the two masses of sample tested

Weight of sample	Fortification level	% Recovery of Vitamins								
		B1	B2	B3acid	B3 amide	B5	B6	B7	B9	C
0.5 g	50%	126	137	125	70.1	88.3	73.9	102	108	145
	100%	73.6	85.7	90	127	100	120	71.1	82.0	98
	200%	134	111	93.4	85.8	64.1	105	97.8	68.5	132
1.0 g	50%	77.2	61.4	103	24.8	54.4	73.6	37.3	42.7	50.4
	100%	65.1	66.9	89.3	25.5	42.6	62.8	62.4	49.8	55.8
	200%	73.3	53.6	102	36.4	42.8	74.7	38.5	40.9	52.7

6.2.2 Selection of buffer

In this section, it was examined whether ammonium acetate or ammonium formate was the most efficient salt as extraction buffer. As shown in Table 15, the results are quite similar for the majority of the analytes. However, it was observed that ammonium acetate was more efficient especially for vitamins B1, B3 acid and B9.Moreover, ammonium acetates enhanced the ionization of the vitamins giving higher intensity (peak areas) for all the vitamins.

Table 15: Results for each buffer salt tested

Buffer	Fortification level	% Recovery of Vitamins								
		B1	B2	B3acid	B3 amide	B5	B6	B7	B9	C
Ammonium acetate (10mM, pH7)	50%	77.3	73.4	64.8	39.1	127	80.9	20.0	55.6	37.1
	100%	115	84.1	75.4	48.2	125	77.1	19.9	64.5	38.8
	200%	97.1	79.3	70.2	38.1	148	70.1	10.2	60.4	38.4
Ammonium formate (10mM, pH7)	50%	253	97.6	48.1	35.0	131	117	23.2	40.5	32.9
	100%	289	57.6	56.1	23.1	101	80.2	21.0	41.9	36.5
	200%	120	61.0	47.1	31.6	167	65.6	12.6	48.0	34.1

6.2.3 pH of buffer

Since WSVs degrade under different pH values, different pH values had to be examined, so as to be selected the most appropriate for the most vitamins. From the results of Table 16, it was concluded that acidic conditions were more

beneficial for the majority of the vitamin expect for vitamins B5 and B7 that prefers the neutral condition. This result wasin accordance with the literature that suggest the acidic conditions for the extraction of WSVs [230]. Although, it was chosen pH 7 as the pH of the ammonium acetate in order to study the efficiency of various acids in the extraction method.

Table 16: Results for each pH value of buffer tested

pH of buffer	Fortification level	% Recovery of Vitamins								
		B1	B2	B3acid	B3 amide	B5	B6	B7	B9	C
3.5	50%	62.2	73.1	83.8	53.6	41.1	67.9	29.8	22.8	41.6
	100%	85.2	96.4	82.8	76.3	35.4	95.8	39.8	13.7	70.1
	200%	58.9	116	143	67.2	48.0	108	29.3	27.6	35.4
4.5	50%	54.8	77.2	87.7	71.1	58.3	41.4	35.2	10.5	48.3
	100%	75.4	41.3	108.0	121	59.6	67.5	39.4	25.4	59.3
	200%	140	72.2	98.7	96.6	51.4	38.8	42.6	24.3	70.0
7.0	50%	65.5	62.0	151	122.0	99.0	78.1	51.8	73.1	630
	100%	89.9	71.0	96.9	65.8	84.3	95.8	53.3	42.9	70.1
	200%	82.5	53.1	111	110	71.1	77.6	54.8	43.9	70.3

6.2.4 Addition of acidic agent

As proved in the section below, the acidic conditions were important for the extraction of WSVs during the sample preparation.For this purpose, six different acid solutions were tested with similar pH values to compare theirefficacy. According to the literature, HCl was the most appropriate acid for the acid hydrolysis of the vitamins B1, B2 and B6 while MPA for vitamin C [230].

From the experiments, it was proved that TCA was the best acid providing the best recoveries for the most vitamins, while perchloric acid was totally inappropriate for the extraction of all the vitamins giving the lower recoveries. As for acetic acid, the % recoveries were not satisfactory for vitamins B3 acid (27.0-54.5%) and C (29.9-42.2%), while MPA was appropriate only for vitamin C providing the best % recoveries (89.7-111%) in comparison to other acid. Comparing TCA and TFA, the % results were almost the same for all vitamins except from vitamin B5 (101-107% and 36.9-39.0% respectively) and vitamin C (50.0-72.9% and 16.9-34.1% respectively). Thus, TCA was considered the best acid for the extraction of WSVs (Table 17).

Table 17: Results for each acidic solution tested

Acids	Fortification level	% Recovery of Vitamins								
		B1	B2	B3acid	B3amide	B5	B6	B7	B9	C
HCl 0.1M	50%	50.3	60.9	34.6	123	34.2	56.2	34.2	15.5	10.2
	100%	54.5	45.3	31.7	28.1	62.4	53.6	28.5	10.6	17.3
	200%	47.0	50.8	24.8	55.6	98.9	43.5	23.9	21.1	25.2
Perchloric acid 0.1M	50%	12.9	1.59	24.9	29.5	21.5	25.5	32.4	8.23	10.9
	100%	12.3	6.55	58.0	28.0	29.4	21.9	16.3	10.8	26.7
	200%	31.9	8.11	12.5	15.8	35.1	19.7	8.10	10.0	19.8
Acetic acid 5M	50%	130	78.9	54.5	40.7	128	68.9	68.4	68.2	36.7
	100%	53.5	139	27.7	60.9	108	92.8	54.0	59.1	42.3
	200%	82.8	133	40.9	38.0	112	90.8	45.4	47.4	29.9
TCA 9.8% w/v	50%	89.6	124	144.0	61.0	107	154	75.0	81.0	50.6
	100%	96.4	114	71.7	72.9	104	220	115	105	67.3
	200%	103	158	120.0	85.2	101	167	138	106	72.9
TFA 9.8% w/v	50%	99.3	105	63.1	61.9	37.2	117	126	183	34.1
	100%	78.9	82.5	55.6	54.5	39.0	87.6	111	139	22.6
	200%	73.7	81.4	57.6	61.3	36.9	76.5	127	122	16.9
MPA 2% w/v	50%	10.6	28.8	28.7	20.9	143	11.0	33.5	49.2	111
	100%	13.4	57.8	31.5	20.1	139	20.1	19.8	49.7	89.7
	200%	10.9	53.5	29.9	21.0	164	17.4	26.5	44.1	101

6.2.5 Concentration of TCA

After the selection of TCA as the most ideal acid for the sample's preparation, it was studied two different concentrations of it (Table 18); 5.0% and 9.8% w/v. The concentration of 5.0% w/v was preferred over 9.8% w/v, because it provided better recoveries except from vitamins B3 amide (59.2-77.0% for 50% w/v and 49.7-61.9% for 9.8% w/v), B5 (73.4-88.8% for 5.0% w/v and 36.6-40.1% for 9.8%) and B7 (100-110% for 5.0% w/v and 55.2-62.3% for 9.8%).

Table 18: Results for each TCA concentration tested

Concentration of TCA (w/v)	Fortification level	% Recovery of Vitamins								
		B1	B2	B3acid	B3 amide	B5	B6	B7	B9	C
5.0%	50%	30.3	75.7	117	77.0	88.8	120	100	76	193
	100%	31.8	118	111	65.5	75.8	112	110	118	197
	200%	38.5	129	115	59.2	73.4	110	110	140	229
9.8%	50%	23.6	124	132	49.7	40.1	103	63.4	145	139
	100%	28.1	113	100	53.8	36.6	101	56.3	121	149
	200%	37.5	110	86.8	61.9	39.3	109	55.2	94	191

6.2.6 Concentration of BHT in MeOH

As it was mentioned in section 5.3.6, BHT is necessary as antioxidant to protectthe vitamins' oxidation during the experimental procedure. Thus, three different concentrations of BHT were studied; 0.1% w/v, 0.5% w/v, and1% w/v. As shown from the results of Table 19, the best recoveries were achieved with 0.5 and 1.0 % w/v BHT for all the vitamins, while in case of 0.1% w/v BHT, the % recoveries were lower. As a result, it was concluded that 0.1% w/v was insufficient to prevent vitamins' oxidation.

Table 19: Results for each BHT concentration

Concentration of BHT (w/v)	Fortification level	% Recovery of Vitamins								
		B1	B2	B3acid	B3 amide	B5	B6	B7	B9	C
0.1%	50%	44.7	69.3	122	147	68.0	82.5	48.4	61.3	58.6
	100%	38.6	68.3	133	125	59.3	72.1	50.3	63.5	51.0
	200%	36.9	50.0	110	111	67.6	67.4	44.6	54.8	49.6
0.5%	50%	75.1	81.0	91.3	94.4	89.6	117	78.8	75.7	81.6
	100%	86.4	76.3	134	81.5	125	115	123	87.6	66.2
	200%	103	91.0	110	92.8	129	112	93	84.3	68.5
1.0%	50%	121	67.1	67.5	75.5	101	74.9	88.6	137	76.3
	100%	118	60.4	97.8	96.8	75.4	89.7	143	95.7	60.0
	200%	170	50.9	124	98.5	70.5	76.1	68.2	127	48.2

6.2.7 Proportion of solvents

After the selection of the most appropriate extraction solvents, it was necessary to investigate their proportion in the solution. Thus, seven occasions were studied. According to the results of Table 20,the most satisfying results were achieved with test C for almost all the analytes by adding 4.5mL of buffer, 3.5mL of TCA and 2mL of methanolic solution of 0.5% w/v BHT to 0.5g of the cereal sample . The

presence of methanolic solution of BHT helped the purification of the sample from interreferences compounds and improved significantly the chromatographic image and especially the shape of the peaks in proportion of 80% aqueous and 20% organic phase. In addition, the proportions of the buffer and TCA affected the total pH value of the extracted solution. It was noticed a difference between the test C and D, where % recoveries of vitamins B2, B3 acid, B9 and C were reduced significantly as shown in Table 20. For this reason, test C was considered the optimal choice of the majority of the analytes.

Table 20: Results for each proportion of buffer: TCA: MeOH

Proportion of buffer:TCA:MeOH (mL)	Fortification level	% Recovery of Vitamins								
		B1	B2	B3acid	B3 amide	B5	B6	B7	B9	C
5:4:1 (A)	50%	45.1	71.5	34	17.3	23.2	47	80.7	75.6	56.2
	100%	39.7	50.9	27.5	47.9	42.9	48.9	68.6	63.7	39.6
	200%	33.2	58.9	21.9	45	64.9	49	67.6	61.3	54.9
5:3:2 (B)	50%	31.1	62.5	71.8	81	116	48.5	59.1	79.3	60
	100%	27.9	66.1	60.8	80.9	125	53.3	63.2	101	32
	200%	25.3	74	55.9	84.9	146	47.8	75.4	94.7	56
4.5:3.5:2 (C)	50%	132	110	97.2	71.7	122	66.7	101	75.6	60.7
	100%	144	102	69.7	125	134	122	75.4	63.7	81.1
	200%	140	91.1	73.7	97.2	89.7	69	72.3	61.3	79
4:4:2 (D)	50%	96.1	65.1	56.8	96.2	97	99.5	70.3	45.1	33
	100%	98.6	51.6	54.4	91.5	153	61.3	71	50.2	30.3
	200%	122	43.3	49.3	105	157	61.6	101	34.5	51.8
4:3:3 (E)	50%	105	84.7	38.3	102	90.1	94.4	63.1	87.1	42
	100%	129	54	85.5	83.4	158	58.6	112	64.5	20.3
	200%	172	49.2	57.8	88	154	62.1	70.6	74.1	37.8
3:5:2 (F)	50%	83.6	76.3	59.7	107	79.8	51.6	56.2	42.4	46.9
	100%	86.7	42.7	41.1	103	58.1	92	50.9	31.1	46.9
	200%	104	45.6	65.7	111	49	56.1	60.6	49.8	53.6
3:3:4 (G)	50%	145	74.4	48.1	86.3	76.1	133	68.7	74.1	36.8
	100%	106	50.2	30.5	58	44.6	103	55.1	78.7	56.8
	200%	120	60.1	56.1	63.2	58.6	116	80.1	84.6	41.2

6.3 Optimization of extraction method

6.3.1 Defatting solvent

To achieve a further clean-up of the samples from lipids and proteins, the existence of a purification step in the method was necessary. For the specific targeted compounds, a strong organic and no water-soluble solvent had to be selected. So, as defatting factors, chloroform, hexane and heptane were tested. Chloroform was selected for this step as it was provided better recoveries for all the vitamins in comparison to hexane and heptane, that their results were almost the same as shown in Table 21. Moreover, chloroform had the advantage to be the lowest phase in contrast to the other organic solvents, fact that facilitated the isolation of the aqueous phase and improved the transparence of the extraction solution.

Table 21: Results of the different defatting solvents tested

Defatting solvent	Fortification level	% Recovery of Vitamins								
		B1	B2	B3acid	B3 amide	B5	B6	B7	B9	C
Chloroform	50%	74.8	77.2	59.2	71.1	58.3	69.2	54.6	77.3	64.2
	100%	75.4	116	46.1	121	59.6	101	67.9	64.7	70.1
	200%	102	72.2	66.5	96.6	51.4	116	68.8	74.3	103
Hexane	50%	62.5	56.0	58.4	80.4	55.1	64.7	55.3	54.8	48.3
	100%	104	65.7	46.4	113	80.6	69.7	45.2	35.8	59.7
	200%	119	69.5	65.9	77.1	62.3	107	40.9	42.5	71.6
Heptane	50%	62.2	25.1	48.3	79.8	58.3	62.0	44.1	39.3	45.2
	100%	56.4	39.9	39.1	69.4	54.2	64.4	39.8	22.4	30.2
	200%	49.4	38.8	62.4	71.2	29.5	53.7	50.1	38.5	38.6

6.3.2 Shaking-sonication sequence

Shaking and sonication as procedures were utilized in order to accelerate and facilitate the extraction of the analytes from the matrix. It was crucial to investigate if the sequence of these extraction techniques affected or not the extraction of the vitamins. For this purpose, samples were analyzed in both two ways and the results are presented in Table 22. In all the cases, the % recoveries were almost the same as, no important differences were observed. The variance of the %recoveries in sonication-shaking sequence was importantly bigger, especially for

vitamins B3 amide (71.9-145%), B6 (136-203%), B7 (116-186%) and B9 (128-191%).

Table 22: Shaking-sonication sequence's results as recoveries

Sonication-shaking sequence	Fortification level	% Recoveries of Vitamins								
		B1	B2	B3acid	B3 amide	B5	B6	B7	B9	C
Shaking-sonication	50%	109	80.3	57.2	77.2	65.4	127	111	116	60.7
	100%	85.8	73.3	59.2	68.2	60.1	127	101	118	64.6
	200%	105	71.7	54.7	59.6	77.3	105	95.5	102	64.2
Sonication-shaking	50%	127	102	40.8	112	66.5	136	186	191	71.0
	100%	104	82.0	65.8	71.9	46.7	189	116	128	55.1
	200%	159	63.7	51.2	145	48.4	203	146	186	79.0

6.3.3 Extraction time

Finally, shaking and sonication time were studied so that to be decidedtheoptimal extraction time of the samples. For that purpose, 10, 15, 20, 25, and 30 min were tested for every procedure and according to results the ideal combination constituted 30 min shaking and 30 min sonication for the samples. As shown in Table 23, it was noticed that the more shaking time was used, the better % recoveries were achieved for all the vitamins. For this reason, 30 min were selected as the optimum shaking time for all the analytes of interest.As for the sonication time (Table 24), it was observed the same tendency even though the improvement of the %recoveries was firstly noticed at 20 min of sonication that could be selected as the best time for sonication.However, there is an increased in the % recoveries especially for vitamin B1 (from 24.0% to 44.6 %), B6 (from 19.3% to 51.7%) and B7 (from 26.3% to 48.1%). Thus, 30 min of shaking and sonication were selected as the best extraction duration.

Table 23: Times of shaking tested

Time of shaking (min)	Fortification level	% Recovery of Vitamins								
		B1	B2	B3acid	B3 amide	B5	B6	B7	B9	C
10	50%	18.6	18.4	17.5	38.0	24.5	10.8	13.5	14.6	23.1
	100%	12.3	27.3	32.9	32.1	12.6	11.4	14.0	14.4	30.1
	200%	12.0	36.9	25.2	45.0	19.4	9.04	19.0	18.4	27.0
15	50%	27.9	43.1	28.8	34.8	37.9	23.5	16.7	21.1	38.1
	100%	16.6	54.6	22.6	48.8	19.6	18.9	18.4	23.0	24.5
	200%	10.8	33.9	19.5	32.0	19.7	15.9	18.8	29.7	34.3
20	50%	25.3	76.8	18.7	49.0	67.8	20.1	27.5	39.0	39.8
	100%	24.0	64.3	17.5	47.8	59.7	19.3	26.3	48.5	47.3
	200%	24.0	54.1	26.3	35.1	64.5	20.3	30.7	37.2	42.0
25	50%	34.0	54.1	17.9	45.9	84.3	40.3	47.4	34.3	36.5
	100%	32.4	72.6	25.7	41.0	82.7	34.6	51.8	41.1	36.6
	200%	15.8	69.8	22.8	32.2	68.7	40.0	31.3	37.8	45.1
30	50%	40.9	80.0	39.4	68.1	80.6	56.9	58.6	54.7	52.0
	100%	44.6	120	31.2	59.6	102	67.3	51.7	48.1	42.7
	200%	49.0	109	31.1	61.2	132	51.1	50.2	47.6	49.4

Table 24: Times of sonication tested

Time of sonication (min)	Fortification level	% Recovery of Vitamins								
		B1	B2	B3acid	B3 amide	B5	B6	B7	B9	C
10	50%	29.1	27.7	34.1	35.4	49.7	30.4	40.3	16.3	27.1
	100%	28.6	22.4	21.9	30.9	60.1	28.5	45.9	31.8	29.2
	200%	15.5	21.8	19.7	28.0	55.0	29.8	47.8	23.8	19.3
15	50%	16.2	31.3	28.2	39.0	68.1	34.7	51.8	25.1	26.1
	100%	19.3	46.2	27.3	25.7	67.8	52.4	53.7	44.1	34.1
	200%	29.3	31.1	30.4	34.0	70.6	58.2	49.9	38.4	35.6
20	50%	33.2	51.4	33.9	47.4	120	44.2	61.8	51.2	40.0
	100%	20.1	56.8	20.1	45.8	80.5	47.9	57.5	40.5	32.8
	200%	40.1	48.6	33.3	49.1	88.2	55.3	68.6	49.8	37.8
25	50%	40.9	72.7	32.2	66.2	111	79.7	59.4	55.6	54.1
	100%	45.0	120	45.3	41.5	85.2	62.9	54.6	59.5	37.1
	200%	35.1	89.4	36.8	53.8	87.6	76.1	60.0	46.1	46.2
30	50%	40.9	80.0	39.4	68.1	80.6	56.9	58.6	54.7	52.0
	100%	44.6	120	31.2	59.6	102	67.3	51.7	48.1	42.7
	200%	49.0	109	31.1	61.2	132	51.1	50.2	47.6	49.4

6.4 Method validation

The fortification levels that have been chosen for proving linearity and precision, correspond to the percentages 10% to 300% of the assigned values of the vitamins in the cereal matrix that is examined. The concentrations of the vitamins, which do not exist in the product (B3 acid, B7 and C), were selected according to literature and cereal products from market.

6.4.1 Identification

The identification of vitamins was evaluated by comparing the retention times of a vitamin of a standard solution and a sample. In Figures 15 and 16 are presentedand compared the typical chromatograms of a standard solution and an analyzed samples. It was noticed that there is not significant difference in the retention times and all the analytes are identified.

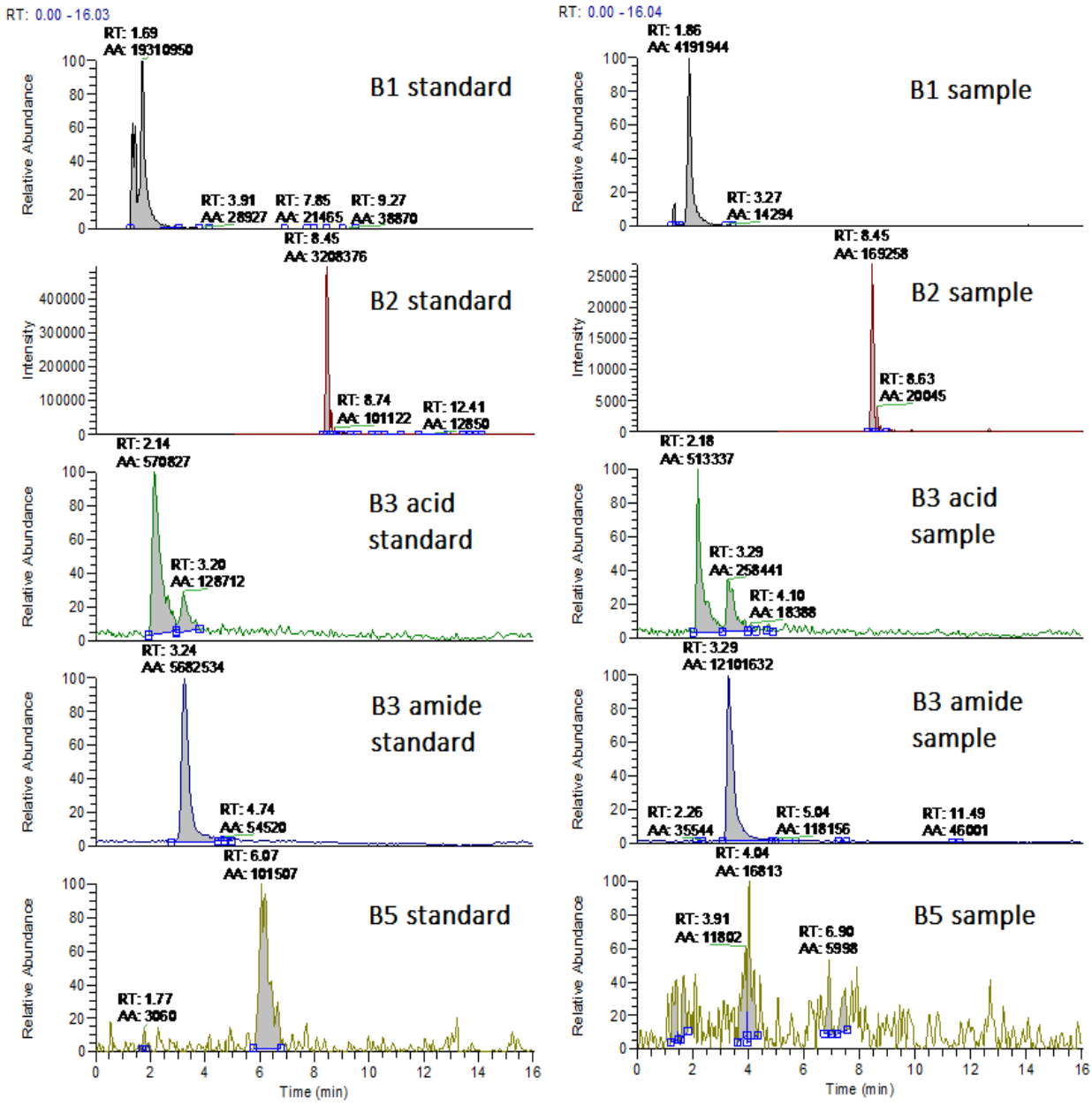


Figure 15: Chromatogram of vitamins B1, B2, B3 and B5 in a standard solution and a sample

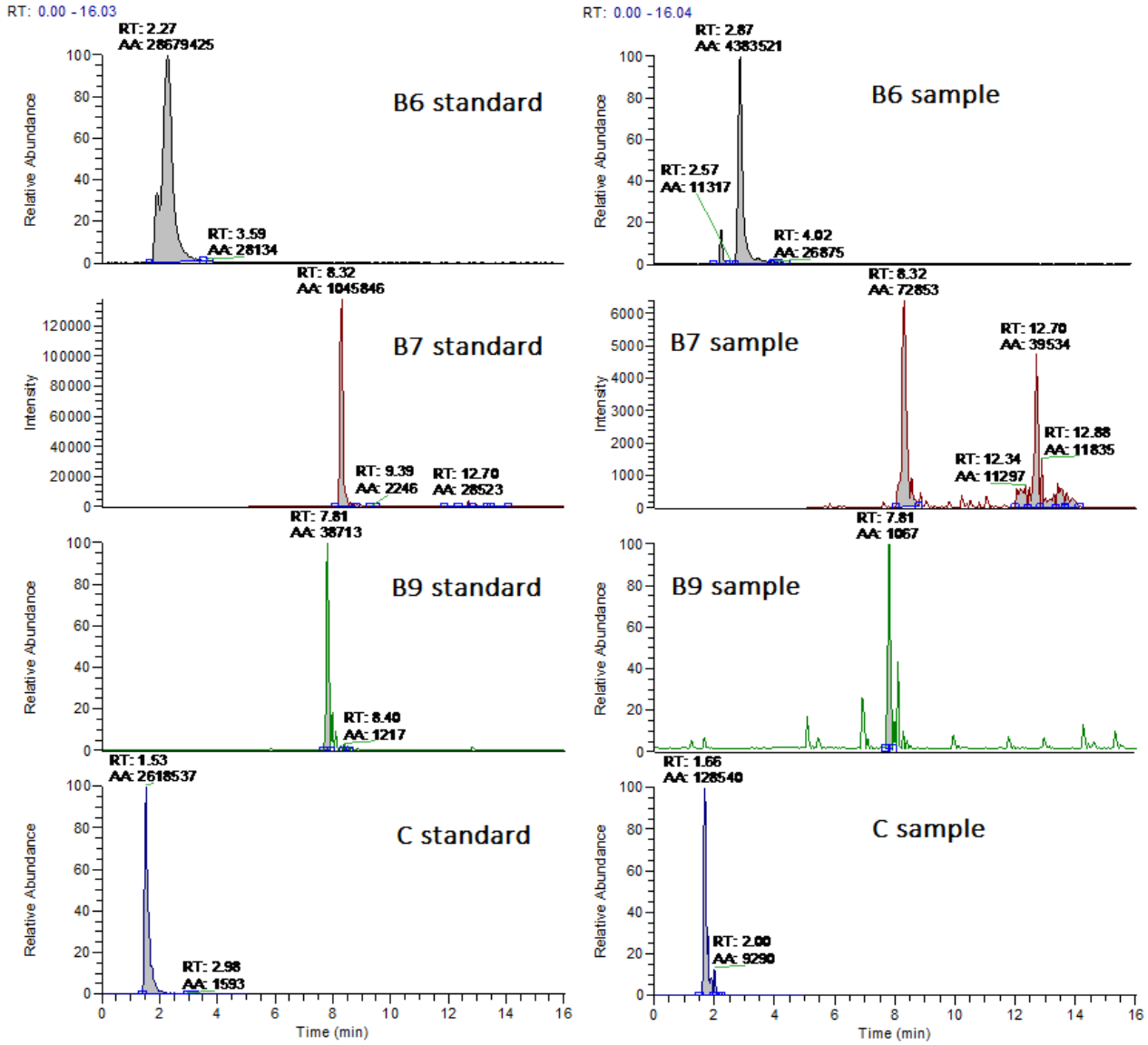


Figure 16: Chromatogram of vitamins B6, B7, B9, and C in a standard solution and a sample

It was also important to calculate and assess the ion ratios of the samples in comparison with the ion ratios of the standard solutions. The ion ratio was calculated as an intensity (or peak area) ratio of a less intense ion to a more intense ion according to the following equation:

$$\%\ \text{Ion Ratio} = \frac{\text{Peak Area Qualifier}}{\text{Peak Area Quantifier}} \text{x} 100\%$$

The result is compared to the relative tolerance limits of the European Commission guidance [228] (Table 25).

Vitamin	Ionratio	Tolerancelimits
B1	51%	±20%
B2	41%	±25%
B3 acid	106%	±20%
B3 amide	53%	±20%
B5	55%	±20%
B6	90%	±20%
B7	6.9%	±50%
B9	45%	±25%
C	84%	±20%

6.4.2 Linearity

The linearity of calibration curves was assessed using a seven-point standard solution calibration curve in pure solvents as well as in blank sample extracts at different concentrations. The linear regression analysis was carried out by plotting the peak area versus the analyte concentrations for compounds with no corresponding IS and the peak area ratio of the analyte and IS versus the analyte concentrations when an IS correction was used. The first type of chart was applied for vitamins B3, B5, B7, B9, and C, while the second one for B1, B2 and B6. The regression line of the form y = bx + a and the correlation coefficients R^2, for spiked and matrix-matched samples were determined. In Tables 26, 27 and 28 are presented the calibration curves of standard solutions ,spiked samples and matrix-matched samples respectively.

It was noticed that the linearity was satisfactory for all analytes in all the cases with the correlation coefficients were >0.98.

Table 25: Standard calibration curves and correlation factors for each analyte

Vitamin	Concentration range (mg/L)(n=7)	Calibration curve	Coefficient of determination (R^2)
B1	0.0750-2.25	y=0.19(±0.63)+8.76(±0.55)x	0.98
B2	0.0750-2.25	y=0.01(±0.13)+2.07(±0.11)x	0.990
B3 acid	0.250-7.50	y=-78194(±83316)+337180(±21659)x	0.98
B3 amide	0.0900-2.70	y=60994(±109324)+7233016(±78946)x	0.9994
B5	0.008-0.225	y=-1485(±2549)+61927(±1104)x	0.998
B6	0.0750-2.25	y=0.63(±0.51)+12.60(±0.44)x	0.994
B7	0.0100-0.300	y=-56403(±50875)+12009176(±330644)x	0.996
B9	0.0110-0.330	y=-10673(±5421)+495675(±29664)x	0.990
C	5.00-150	y=328843(±104812)+46878(±1362)x	0.995

Table 26: Standard addition calibration curves and correlation factors for each analyte

Vitamin	Concentration range (mg/kg)(n=7)	Calibration curve	Coefficient of determination (R^2)
B1	1.50-45.0	y=3.86(±0.15)+0.0779(±0.006)x	0.98
B2	1.50-45.0	y=0.080(±0.033)+0.0177(±0.0014)x	0.997
B3 acid	5.00-150	y=-290772(±280714)+131404(±3649)x	1.00
B3 amide	18.0-540	y=14097751(±4717305)+208722(±17032)x	0.991
B5	0.150-4.50	y=8032(±4566)+40118(±1978)x	0.990
B6	0.150-4.50	y=0.657(±0.542)+0.806(±0.021)x	0.997
B7	0.200-6.00	y=112697(±27678)+196287(±8994)x	0.991
B9	0.220-6.60	y=13434(±1692)+7494(±439)x	0.98
C	100-3000	y=-1712(±36356)+666(±24)x	0.994

Table 27: Matrix-matched calibration curves and correlation factors for each analyte

Vitamin	Concentration range (mg/L)(n=7)	Calibratio ncurve	Coefficient of determination (R^2)
B1	1.50-45.0	y=5.49(±0.21)+0.218(±0.009)x	0.991
B2	1.50-45.0	y=-0.189(±0.049)+0.0529(±0.0021)x	0.992
B3 acid	5.00-150	y=2114204(±151425)+130437(±1968)x	1.00
B3 amide	18.0-540	y=5921169(±438529)+345351(±19000)x	0.990
B5	0.150-4.50	y=-33947(±11250)+60280(±4874)x	0.990

B6	0.150-4.50	y=16.1(±0.501)+0.546(±0.0217)x	0.992
B7	0.200-6.00	y=-69403(±90403)+467241(±29377)x	0.994
B9	0.220-6.60	y=782(±2863)+19365(±846)x	0.991
C	100-3000	y=105234(±60343)+731(±39)x	0.990

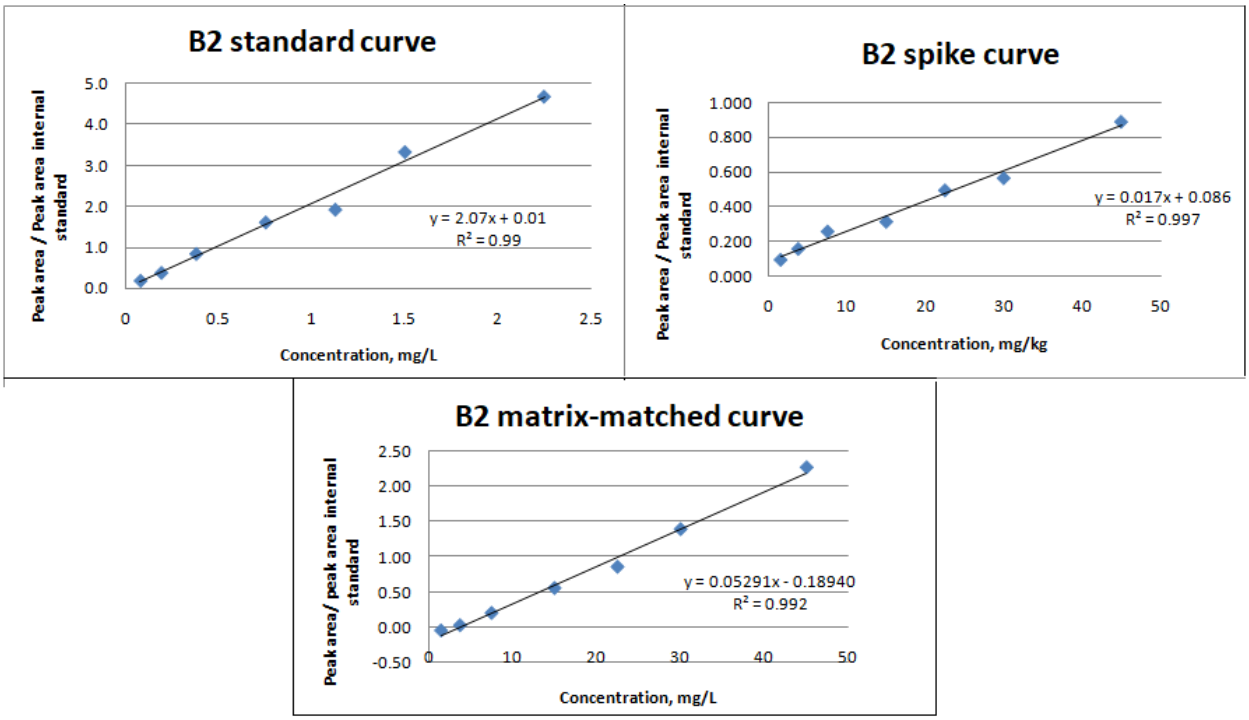


Figure 17: Standard, standard addition and matrix-matched calibration curves of B2

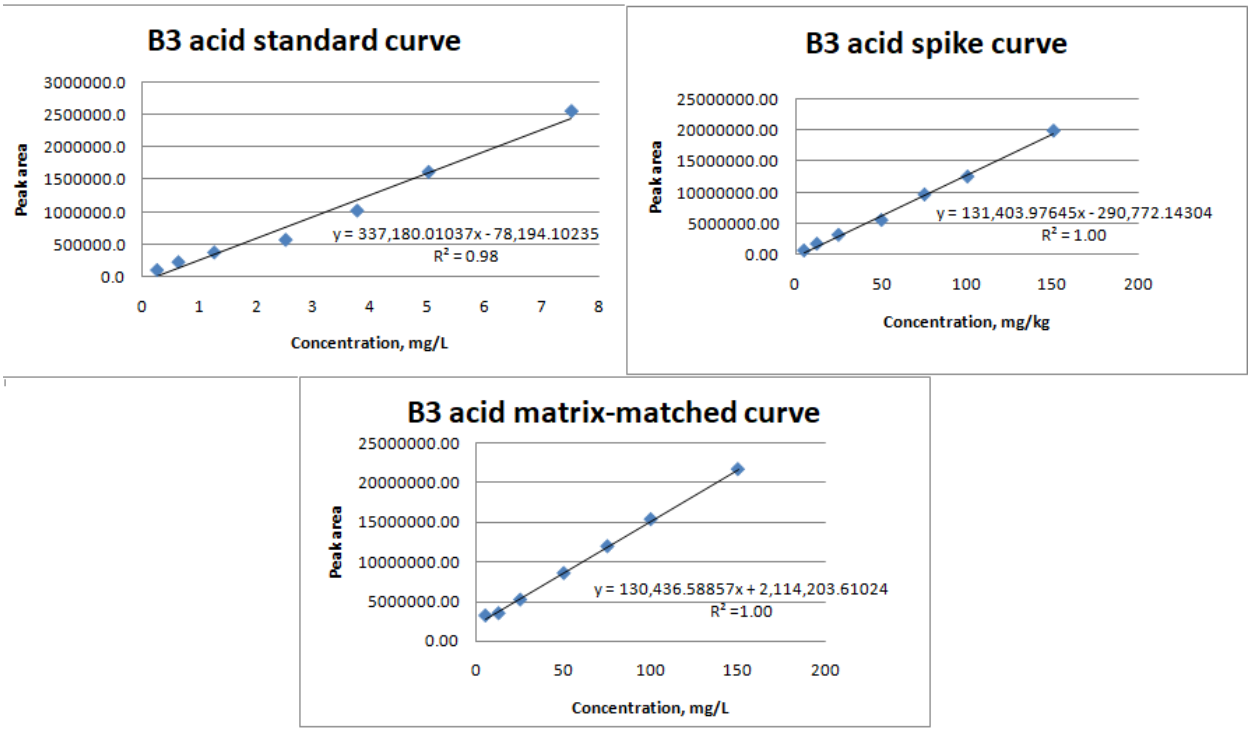


Figure 18: Standard, standard addition and matrix-matched calibration curves of B3 acid

6.4.3 Precision

The precision of this method was calculated from the intra-day precision (repeatability). Precision results for all compounds in the three fortification levels are presented in Table 29. It can be observed that %RSD values were lower than 18% and the % recovery ranged from 83.2% to 118% for the vitamins in the three fortification levels.These results demonstrated good precision and repeatability for the developed methodology.

Table 28: Comparative results of recoveries and precision for each analyte

Vitamin	Level A				Level B				Level C			
n=6	Mean Concentration (mg/kg)	Mean Recovery (%)	SD	%RSD$_r$	Mean Concentration (mg/kg)	Mean Recovery (%)	SD	%RSD$_r$	Mean Concentration (mg/kg)	Mean Recovery (%)	SD	%RSD$_r$
B1	6.9	91.6	1.1	15.4	17.16	114	0.97	5.63	29.4	98.1	2.7	9.01
B2	6.51	86.8	0.55	8.43	11.31	83.2	0.48	4.21	34.5	115	1.7	4.91
B3 acid	25.9	104	3.8	14.7	46.7	93.4	5.7	12.2	118.5	118	2.2	1.88
B3 amide	44.8	100	7.3	16.2	158	88.2	11	7.18	358	99.5	50	14.1
B5	0.71	94.6	0.11	16.1	1.62	108	0.24	14.6	2.94	98.0	0.43	14.7
B6	6.43	85.8	0.41	6.41	16.8	112	1.3	7.80	28.3	94.4	3.2	11.2
B7	0.95	94.9	0.15	15.6	2.20	110	0.39	17.8	4.09	102	0.78	19.0
B9	1.13	102	0.17	15.5	2.32	105	0.31	13.4	3.89	88.4	0.39	9.92
C	536	107	55	10.2	951	95.1	159	16.8	2017	101	323	16.0

6.4.4 LODs & LOQs

LODs and LOQs were calculated as described in section 5.5.6. The obtained results are presented in Table 30. It was noticed that the instrumental as well as the methods' LODs and LOQs were satisfying. More specifically , it was observed that for vitamins B1, B5 and B6, the instrumental LODs were almost similar to the methods' LODs. The difference between the instrumental and method's LODs was remarkable for vitamin B3 acid, B3 amide and C. This may be occurred due to the high concentrations of the vitamins in the matrix.

Table 29: Instrumental and Method's limit of detection (LOD) and limit of quantification (LOQ) of each vitamin

Vitamin	Instrumental		Method	
	LOD (mg/L)	LOQ (mg/L)	LOD (mg/kg)	LOQ (mg/kg)
B1	0.112	0.335	0.206	0.618
B2	0.141	0.423	1.30	3.90
B3 acid	0.408	1.22	3.39	10.3
B3 amide	0.170	0.510	2.82	8.47
B5	0.0906	0.272	0.109	0.333
B6	0.0921	0.276	0.0922	0.276
B7	0.0140	0.0419	0.215	0.652
B9	0.0489	0.147	0.288	0.872
C	7.38	22.1	20.7	62.9

6.4.5 Matrix effect

Percentages higher than 20% or lower than-20%, are indicative of a strong matrix effect. The %ME, calculated as described in 5.5.7, are presented in Table 31.It was observed strong matrix suppression for all the studied compounds, as it was expected due to the complexity of the matrix and interfering compounds.

Table 30: Matrix effect for each analyte in LC-MS/MS analysis

Vitamin	%Matrixeffect
B1	-96.1
B2	-94.2
B3 acid	-94.1
B3 amide	-95.2
B5	-93.1
B6	-95.4
B7	-94.7
B9	-94.1
C	-99.5

6.4.6 Application to real samples

The optimal method was applied to two real samples, so as to be evaluated as far as concerned its reliability. The results were satisfying for both samples with recoveries 67.8-100% for classic cereal and 93.0-149% for cereal with fruits, as shown in Table 32. The results are complied with the tolerance limits according to the European Commission guidance [9].

Table 31: Results for real samples in comparison with the tolerance limits

Vitamin	Label'svalue (mg/kg)	Tolerance limits		Experimentalconcentration (mg/kg)	
		-35%	+50%	Classiccereal	Cerealwithredfruits
B1	18.0	11.7	27.0	13.5	16.7
B2	23.0	15.0	34.5	15.6	25.5
B3 acid	134	87.1	201	134	135
B3 amide	134	87.1	201	106	151
B5	-	-	-	-	-
B6	12.0	7.8	18	10.8	16.8
B7	-	-	-	-	-
B9	3.34	2.17	5.01	3.07	4.98
C	-	-	-	-	-

6.5 Conclusions

Vitamins are widely added in both food matrices and pharmaceutical preparations, in order to coverthe daily needs and prevent or treat diseases

caused from their lack. It is known that vitaminscan be degradedunder specific circumstances. Thus, foodstuffs and pharmaceutical products should be controlled, so that their vitamin's content are in accordance with their labelled values.

The goal achieved through this study was the development of a sensitive and reliable methodology for the simultaneous determination of water-soluble vitamins in an extremely complex matrix such as the breakfast cereals. The final sample preparation procedure comprised of a liquid-liquid extraction of the matrix facilitated by shaking and ultrasound waves, followed by centrifugation and filtration. This method yielded the best results recovery- and matrix effect- wise and was validated according to the EC/2002/657 Regulation. The obtained %RSD, LOD and LOQ values were deemed acceptable for the 7 vitamins. Still, as evidenced by these parameters, the developed methodology is not suitable for certain analytes, for whom perhaps more specific and extensive sample treatment must be applied.

The future plans for the extension of the study could be the optimization of method for the determination of vitamin B12 in cereals. Moreover, this method can be tested, optimized and validated in other food matrices such as dairy products, baby foods, honey as well as pharmaceuticals and animal feeds. Finally, the accuracy of the methods can be tested with the participation in proficiency tests.

ABBREVIATIONS-ACRONYMS

AA	Ascorbic acid
ACN	Acetonitrile
AI	Adequate intake
APCI	Atmospheric pressure chemical ionization
API-MS/MS	Atmospheric pressure ionization tandem mass spectrometry
BHT	Butylated hydroxytoluene
CE	Collision energy
CE	Compact electrophoresis
CoA	Coenzyme A
CVD	Cardiovascular diseases
DAD	Diode array detector
DHAA	Dehydro-L-ascorbic acid
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EC	European Commission
ESI	Electrospray ionization
EU	European Union
FA	Formic acid
FAD	Flavin adenine dinucleotide
FAO	Food and Agriculture Organization
FIA	Flow injection analysis
FLD	Fluorescence detector
FMN	Flavin mononucleotide
FT-ICR	Fourier transform - Ion cyclotron resosnance
GABA	Gamma-aminobutyricacid
GC	Gaschromatography
HILIC	Hydrophilic interaction chromatography
HIV	Human immunodeficiency virus
HPLC	High pressure liquid chromatography
HRMS	High resolution mass spectrometry
IAA	Isoascorbic acid
ILIS	Isotope labelled internal standard
IS	Internal Standard
LC	Liquid chromatography
LC-IDMS	Liquid chromatography - Isotope dilution mass spectrometry
LC-MS/MS	Liquid chromatography tandem Mass Spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
MCC	3-Methylcrotonyl-CoA carboxylase

ME	Matrixeffect
MeOD	Deuterated methanol
MeOH	Methanol
MPA	meta-Phosphoric acid
MRF	Minimally processed food
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MSC	Multisyringe chromatography
NA	Nicotinic acid
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NM	Nicotinamide
NMR	Nuclear magnetic resonance
NTDs	Negleted tropical diseases
PCC	Propionyl-CoA carboxylase
PDA	Photo diode array
PDHO	Potassium dihydrogen orthophosphate
PLP	Pyridoxal 5' -phosphate
PMP	Pyridoxamine 5' -phosphate
PNP	Pyridoxine 5' -phosphate
PT	Proficiency test
PVDF	Polyvinyl difluoride
QqQ	Triple quadrupole
RC	Regenerated cellulose
RDA	Recommended dietary allowance
RFS	Refeeding syndrome
RNA	Ribonucleic acid
RP-LC	Reversed-phase liquid chromatography
RSD	Relative standard deviation
SAM	S-adenosylmethionine
SPE	Solid phase extraction
SRM	Selected reaction monitoring
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TLC	Thin-layer chromatography
TOF	Time of flight
UK	United Kingdom
UPLC	Ultra pressure liquid chromatography
USA	United States of America
UV	Ultraviolet
WHO	World Health Organization
WSVs	Water-soluble vitamins

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