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ENDOCRINE DISRUPTORS IN FOOD CONTACT MATERIALS; IS THERE A HEALTH THREAT?

Kazimiera Ćwiek-Ludwicka [1], Jan K. Ludwicki [2]*

[1] National Institute of Public Health - National Institute of Hygiene, Warsaw, Poland
[2] National Institute of Public Health - National Institute of Hygiene, Department of Toxicology and Risk Assessment, Warsaw, Poland

ABSTRACT

Food Contact Materials (FCMs) are a major source of endocrine disrupting chemical substances (EDCs), thus forming an important part of human exposure to these compounds, to which this article is addressed. The potential impact of such exposures on endocrine function, and thereby health outcomes, requires scientifically valid evidence so that appropriate risk management decisions can be taken to diminish human exposure, particularly in vulnerable population groups like infants and small children. Relevant aspects of exposure assessment are discussed based on testing migration of EDCs from FCMs, together with the different approaches so used. The specific migration testing determines whether limits for defined substances are met. However not all EDCs present in the leachate may be found by these means. In fact, the chances of detecting EDCs in the food simulant (leachate) are improved when it is subjected the relevant biological testing, thus helping to provide improved protection against these chemical substances. Nevertheless, official controls and risk management decisions do not necessarily take such testing into account, as the relevant legislation is based on specific migration limits that may be easily quantified and addressed in the risk management process. Elucidating the link between observed endocrine activity and any toxic effects so arising, is complicated by the complexity of endocrine interrelationships coupled with relatively limited sensitivity of toxicological tests. Any risk assessment implies a rather high uncertainty and should include also any cumulative effects. This review discusses the effects of the EDCs like bisphenol A, phthalates and benzophenone found in FCMs. In addition, the approaches from the USA and EU for systematically evaluating man-made EDCs in the environment are also considered, including appropriate prioritisation criteria.

Key words*: endocrine disruptors, food contact materials, food packaging, exposure assessment, risk assessment, benzophenone, bisphenol A, phthalates, substances migrating/leaching from food packaging*

STRESZCZENIE

Materiały do kontaktu z żywnością (ang. food contact materials, FCMs) stanowią istotne źródło substancji zaburzających funkcjonowanie układu hormonalnego określanych jako endocrine disrupting chemicals (EDCs). FCMs mają ważny udział w całkowitym narażeniu człowieka na te substancje. Potencjalny wpływ EDCs na funkcjonowanie układu hormonalnego i skutki zdrowotne wynikające z narażenia na te substancje, dostarczają potwierdzonych dowodów do podejmowania decyzji w ramach zarządzania ryzykiem, zmierzających do zminimalizowania narażenia na te związki, co ma istotne znaczenie, zwłaszcza w przypadku grup populacji szczególnie wrażliwych, takich jak niemowlęta i małe dzieci. Omówiono niektóre aspekty oceny narażenia na podstawie badania migracji EDCs z materiałów do kontaktu z żywnością, w zależności od zastosowania różnych metod badania migracji. Badanie migracji specyficznej umożliwia sprawdzenie czy spełniane są limity migracji ustanowione dla poszczególnych substancji. To podejście stwarza ryzyko, że nie wszystkie migrujące EDCs zostaną wykryte. Zastosowanie odpowiednich testów biologicznych do analizy płynu pomigracyjnego stwarza większe prawdopodobieństwo wykrycia obecności EDCs zapewniając lepszą ochronę konsumenta przed tą grupą związków. Jednakże wyniki takich badań nie zawsze umożliwiają podejmowanie decyzji przez urzędową kontrolę w ramach zarządzania ryzykiem, ponieważ większość przepisów opiera się o limity migracji specyficznej, które łatwo mogą być skwantyfikowane w procesie zarządzania ryzykiem. Wyjaśnienie zależności między zaobserwowanym wpływem na układ hormonalny a wystąpieniem szkodliwego skutku działania napotyka na trudności wynikające z ogromnej złożoności wzajemnych zależności w układzie hormonalnym i ograniczonej czułości testów toksykologicznych. To z kolei implikuje stosunkowo dużą niepewność oceny ryzyka, która powinna także uwzględniać możliwość wystąpienia efektów skumulowanych. Przedyskutowano aspekty związane z bisfenolem A, ftalanami i benzofenonem, jako EDCs występującymi w materiałach do kontaktu z żywnością.

***Corresponding author**: Kazimiera Ćwiek-Ludwicka, National Institute of Public Health – National Institute of Hygiene, 24 Chocimska str., 00-791 Warsaw, Poland, phone: +48 22 54 21 266, fax: +48 22 8493513, e-mail: cwludwicka@pzh.gov.pl

Przedstawiono również podejścia USA i UE do systematycznej oceny antropogennych EDCs w środowisku, z uwzględnieniem kryteriów umożliwiających ustalanie priorytetów.

Słowa kluczowe: *endocrine disruptors, materiały do kontaktu z żywnością, opakowania żywności, ocena narażenia, ocean ryzyka, bisfenol A, ftalany, beznozfenon, substancje migrujące z opakowań żywności*

INTRODUCTION

Materials and articles intended to contact with food (Food Contact Materials; FCMs), including food packaging, are not generally perceived to be a chemical health threat when compared to pesticides, veterinary drugs, heavy metals or mycotoxins that are well recognised food contaminants arising from agricultural practices, the environment or improper food storage. However within the last decade, it has become accepted that FCMs are important contributors to human xenobiotics' exposure [27] due to the worldwide debate on such substances like bisphenol A and their impact on the endocrine system. This underpins risk management decisions for placing limits of human exposure to this compound, particularly when directed towards vulnerable groups like infants and small children.

The high migration of phthalates observed from FCMs into foodstuffs required the European Food Safety Authority (EFSA) to undertake a new risk assessment and to establish legally binding FCM limits [45]. In recent times, the Rapid Alert System for Food and Feed (RASFF) notifications on FCMs have increased concerning unacceptable migration levels from FCMs of permitted substances as well as those not allowed [9, 46].

The term 'endocrine disruptors' has recently been substituted by 'endocrine disrupting chemicals' (EDCs) [53] and will henceforth be referred to in this article since it concerns only chemicals and not any other endocrine risk factors. These EDCs, i.e. chemical substances that interfere with endocrine system homeostasis are often defined as xenobiotics, which after being ingested by humans or animals cause unusual endocrine activity resulting from an interaction with relevant receptors. Some definitions further describe the toxic effects on exposed organisms, its progeny or human populations [57]. Extending this definition seems relevant for distinguishing between noxious effects that suggest a toxic response as opposed to rather mild, modulatory activity which may or may not result in a toxic response from the organism.

EDCs mechanisms of action are exceptionally diverse and exposure responses may involve many tissue, organs or system functions. Disorders endocrine system function may due to over-stimulation or depression, resulting in an excessive or insufficient secretion of hormones.

Numerous chemical substances may affect the endocrine system such as phytoestrogens, however the majority of environmental EDCs are both produced and introduced into the environment by human activity. EDC effects on man may be thus considered as a complex response due to interactions of numerous natural and synthetic chemical substances with target receptors in different tissues and organs [29].

Many studies have investigated and reviewed the effect of EDCs on the environment and man [8, 13, 53]. When the relevance of these exposures are evaluated, serious difficulties arise because of the many different factors or variables involved, which include assessing the whole spectrum of possible endocrine responses.

In all the biological response to EDCs are complex due to the many relationships and feedbacks between organs and glands that serve to preserve the organism's homeostasis.

EXPOSURE ASSESSMENT

Dietary exposure to chemical substances from food packaging and other food contact materials may occur as a result of migration from the packaging materials into the food products.

Migration tests of chemical substances from FCMs into foodstuffs or food stimulants, which mimic the eluting properties of food, are used for evaluating consumer exposure to these substances. Since the exposure may be defined as a function of the amount of the substance that migrates into the food and the amount of this food consumed, it may be evaluated indirectly by quantifying a given migrating substance into the food or food simulants specially developed for this purpose. The migration level of substance depends on the kind of packaging material itself, the chemical nature of the foodstuffs having the contact, the concentration of the substance in the packaging material, time and temperature and also on the ratio to the surface area of the packaging material to the amount/volume of the food product.

Migration data for plastic food contact materials may be obtained from monitoring of chemical substances in food or from migration testing into food simulants. Because of the analytical difficulties resulting from complexity of food matrixes migration data are obtained from migration experiments using the food

simulants [47]. The diffusion model for the estimation of the migration of substances from the plastic materials has been also legally allowed for the compliance checking with the specific migration limit (SML).

For assessing the exposure to chemical substances present in packaging it is necessary to know what type of food is packed in what kind of material. Methods proposed to be used in the UE for testing of substances migrating from plastic FCMs have been described and reviewed in detail by *Cwiek-Ludwicka* et al. [11]. One of the major disadvantages of methods for specific migration is that they are designed for quantifying a particular chemical amongst the many others which are usually present in the food simulant used. This is especially important, since the leachates normally found in these studies depend on the materials tested which may contain many potentially toxic chemical substances. It is thus obvious that specific migration testing only focuses on specific substances and does not provide information on any other substances present that may be toxic.

Such a limitation should be taken into account for FCMs safety evaluations since specific migration tests for a given substance are used in the control and monitoring to check that food packaging are in line with legislative provisions rather than to assess actual consumers' exposure. This weakness was documented by *Wagner and Oehlmann* [56] who showed that the leachate from polyethyleneteraphtalate (PET) bottles in a migration study contained unidentified substances with oestrogenic potential, thus providing convincing evidence that specific migration tests should not be the only base for giving an opinion on product safety.

From the toxicological point of view, migration testing in conjunction with biological tests is more appropriate, since it provides better information on whether the substances migrating from the FCMs into foodstuffs may pose a health risk. Moreover, it also facilitates hazard characterisation. Nonetheless, such approach may create legal problems, since, as aforementioned, the legislation that has been designed to facilitate risk management in FCMs are based on the migration limits into foodstuffs or food simulants for defined substances [47]. It may therefore be expected that any risk assessment based on exposure to particular substances migrating from FCMs will bear some uncertainty. One of the possible solutions to such instances might be in setting the cut-off criteria for FCMs whenever the post-migration liquid exhibits hormone disrupting potential.

Exposure to EDCs may be extremely serious during the perinatal period as the foetal endocrine system and that in the later of the new-born is extremely susceptible to chemical stress. Such changes during early stages of development may induce irreversible effects that only emerge in later life [8, 18, 39, 51].

Bisphenol A provides a good example of complex receptor interactions as demonstrated by *in vitro* studies showing it to be both an oestrogenic receptor agonist and an androgenic receptor antagonist [6]. *In vivo* studies also noted many different responses suggesting a potential endocrine effect that was however expressed above its threshold value, i.e. 5 mg/kg body weight (bw) per day [23, 31, 36].

The quantitative exposure assessment of the general population to chemical substances migrating from the FCMs is based on the data concerning consumption of food into which the substance migrates and the magnitude of this migration. The uncertainty resulting from the exposure assessment results in this case from the fact that the food simulants do not always reflect the actual migration that occurs into the real food and not always reflects the worst case scenarios and, moreover that the conventional method for exposure assessment are not adequate for children. In addition, the exposure resulting from the migration into the dry food is generally underestimated [38]. Using food simulants for migration testing, nonetheless allows various hypothetical exposure scenarios to be developed from which a deterministic risk assessment can be performed, so that appropriate preventive measures can be implemented.

There are several approaches to assess exposure to the xenobiotics migrating from the FCMs into the food, and the choice of the right one may depend on the purpose of the assessment. If the purpose is to approximate the actual exposure to a particular substance, the evaluation should be based on the biomonitoring results of this substance or the relevant exposure indicators in the human specimens. Due consideration should be given that this method covers the exposure from all sources leading to a possible overestimation of the exposure from the foodstuff if the substance is also present in other parts of the environment. Another weakness of this method may occur if the substance is rapidly eliminated from the organism and so large fluctuations in short time intervals may occur. This may be partially limited by increasing number of samples tested.

In evaluating product safety, exposure assessment is more relevant for testing FCMs migration into food stimulants as this provides expected real life conditions, enabling different modelling methods to be used and tailored according to the conditions of contact. The more detailed aspects of these two approaches were discussed elsewhere [32].

RISK ASSESSMENT

As mentioned previously, the action of chemical substances affecting the endocrine system is by their ability to hinder or to facilitate functioning of one or

more elements that constitute the endocrine system. It does not necessarily mean that in such cases any adverse effects can arise. The distinguishing the endocrine modulatory activity which does not necessarily cause toxic changes from the activity that triggers the toxicity resulting from the damaging one or more elements of endocrine system is important in the evaluation of potential risk for human health. It is generally accepted that there are threshold levels of exposure below which a given organism is not expected to produce any toxic response [14]. However, due to the rather limited sensitivity of toxicological tests, establishing such thresholds leads to considerable uncertainty. Thus, an important part of any evaluation should determine the relationship between endocrine activity and toxic effect. In the case of an endocrine impact, the mechanisms maintaining homeostasis, protecting the cell against harmful xenobiotics effects should also be taken into account. However, in itself, mobilising such mechanisms may indicate that the xenobiotic has already reached the target cell and induced a protective response. In certain developmental stages of an organism, the ability to induce homeostatic maintenance may be limited resulting in elevated susceptibility of the organism [19] which in the case of perinatal exposure, may be of special concern. In such instances, the precautionary principle approach for risk management seems to be justified, all the more so if currently proposed tests for the endocrine activity in mammals [43, 44] do not include the effects of perinatal exposure, which hitherto emerge in later life stages. The possibility of such delayed effects was indicated by *Betancourt et al*. [4], who found a positive relationship between intrauterine exposure to BPA and elevated susceptibility for mammary carcinogenesis induced by 7,12-dimethyl(a)anthracene in the rats. A similar, but greatly worrying relationship was observed by *Markey* et al. [33] who showed that even small exposures of pregnant mice to BPA altered the development and tissue organisation in the mammary glands of the progeny.

When undertaking risk assessment, the possibility of cumulative effects arising should also be taken into account when the organism may be exposed to more than one substance of a common mode of action or when the toxic effect is the same but results from different modes of action [49]. This situation is very alike to when numerous environmentally persistent organochlorine anthropogenic compounds may contribute to cumulative effects [50].

Risk assessment for EDCs exposure is additionally challenging due to the complexity of interrelationships within the endocrine system that result in chemical substances acting at different endocrine system sites or expressing an affinity to different receptors that may cause the same final effect. This phenomenon was discussed by *Datson* [12] who drew special attention to the uncertainty inherent in evaluating potential exposure effects to mixtures of EDCs. For this reason the opinion that in case of exposure to multiple ECDs, their potential to produce similar effects should rather be taken into account than the modes of their action seems to be justified [29].

ENDOCRINE DISRUPTING CHEMICAL SUBSTANCES IN FOOD CONTACT MATERIALS

During last decade many chemical substances hitherto recognised as safe have now been found to adversely affect hormonal balance in organisms. They include bisphenol A, phthalates, benzophenone and its derivatives [3] along with organic compounds of tin [41] that migrate from FCMs into foodstuffs. The migration of potential EDCs into foodstuffs has stimulated efforts for developing a uniform approach, specially tailored for their identification and evaluation. *Muncke* [37] has listed 50 chemical substances authorised in food contact materials which are known or potential endocrine disruptors.

The endocrine system regulates most of an organism's biological function and there are a vast number of receptor sites, within a complex milieu complex of interrelationships where, following a chemical stimuli, the system may become disrupted. For this reason the indication of the tests that would allow classifying substances as EDCs is very difficult [26]. In case of pesticide residues in foodstuffs that are of special concern, a strategy and temporary criteria for undertaking procedures preceding risk management have been proposed by *Max-Stoeltig* et al. [35]. This takes into account consumers' safety, and proposed two approaches for classifying chemical substances as EDCs. The first approach utilises exposure assessment through determining the amount of substance which enters the organism *via* food, whilst the second approach evaluates and reports the endocrine disrupting potential of the given substance. However, *Rudén* [48] stress that the proposed approaches should be treated as temporary, until the science based criteria enabling the risk assessment to be performed are developed according to EU regulations. It may be anticipated that such criteria would also be relevant for the chemical substances that migrate from FCMs into foodstuffs.

The diversity of materials intended to contact with foodstuffs results from the numerous functions they are designed for. By implication, this also leads to a diversity of problems for evaluating whether, and under which circumstances, a given material may safely be used for making contact with foodstuffs and when the risk becomes unacceptable. One of the major purposes

of food packaging (excluding any marketing role) is to ensure appropriate protection against external factors such as chemical and biological contaminants, preventing oxidation by atmospheric oxygen, light, loss of gas from beverages, loss or absorption of humidity and aroma, etc. In some instances, basic packaging material may also adversely affect the health quality of the packed product, for example metal cans that require internal lacquer coatings to prevent any direct contact of food with the metal surface thus constitute a risk of lacquer components migrating into the food.

Currently used food packaging is made from different plastic materials and numerous laminates. Moreover, the packed foodstuff may come into contact with the internal walls of cans, gaskets and coatings used in lids, which may be a source of harmful or inadequately tested substances [10, 45, 46]. This particularly concerns EDCs which have been exhaustively detailed by *Muncke* [37] who demonstrate that food packaging may indeed contain numerous substances suspected of acting as EDCs. Since foodstuffs may interact with the internal surface of packaging, a migration of its constituents may be expected. Migrating substances may be expected to include monomers, polymerisation initiators, catalysers and numerous other chemical ingredients as well as polymer degradation products like nonylphenol, and also other substances that are intentionally added during production and food processing [7, 25]. Ter *Veld* et al. [54] showed oestrogenic potency of 21 food-packaging-associated compounds, including bisphenol A, nonylphenol and antoxidants such as butylated hydroxyanisole (BHA) and propyl gallate. Examples of endocrine disrupting chemical substances, their role in food contact materials and modes of action are presented in Table 1.

So far there are no internationally recognised classification or evaluation criteria for EDCs for foodstuffs, which could be used as a base for risk assessment. In pesticides for example, the European Commission anticipating new criteria for allowing EDC identification, proposed, (in the Regulation (EC) 1272/2008), that those substances classified as carcinogenic (Category 2) and/or toxic for reproduction (Category 2) should also be treated as endocrine disruptors [21].

In the following are described some examples of substances formerly used in FCMs together with their toxicological evaluations which resulted in their use becoming limited/restricted due to the legal decisions arising from risk management. When describing these substances their functional role in the technological process was ignored.

Bisphenol A (BPA)

This was found in FCMs made of polycarbonate and internal lacquer coatings in metal cans. Toxicological studies on this compound [27, 28, 52] allowed the No Observed Adverse Effect Level (NOAEL) to be established as 5 mg/kg bw/day, and consequently a Tolerably Daily Intake (TDI) level found to be 0.05 mg/kg bw/day.

From the toxicological evaluation report on BPA, EFSA concluded that exposure to relatively high doses, well above 5 mg/kg bw/day, may be related to some estrogenic effects [16]. This value was confirmed in the later scientific opinion where it was shown that even worse, but still realistic exposure scenarios, the safety

Table 1. Endocrine-disrupting substances in food contact materials (FCMs)

Compound name	Role in FCMs	Mode of action toxicological endpoint
Benzophenone	Additive - photo initiator UV to printing inks used for printing cardboard food packaging	Weak estrogen, binds to estrogen receptor
Bis(2-ethylhexyl) phthalate (DEHP)	Additive - plasticiser in plastic foils, resins, PVC hoses, tubing, foams and plastic kitchenware	Affects reproduction and fertility in 2-generation studies
Dibutyl phthalate (DBP)	Additive – plasticiser	Estrogen
Bisphenol A (4,4'-dihydroxy-2,2--diphenylpropane)	Monomer, starting compound in epoxy resins, lacquer coatings of internal surfaces of cans, polycarbonate plastic materials, thermal papers	Estrogen, binds to estrogen receptor
Butylated hydroxyanisole (BHA)	Additive - antioxidant	Estrogen in α and β cell lines of human osteoblasts
Cadmium	FCM contaminant	Activates estrogen receptor
Dimethyltin bis(isooctyl mercapto-acetate)	Plasticiser	Affects 17β-estradiol biosynthesis
Lead	FCM contaminant	Affects reproductive system
Perfluorooctanoic acid (PFOA)	Surface coatings, food containers surfaces	Alteration of thyroid hormone levels
Propyl gallate	Additive- antioxidant	Estrogen in α and β cell lines of human osteoblasts
Semicarbazide	Twist-off type closure internal coatings	Endocrine disrupting potential not confirmed
Thiram	Rubber vulcanisation accelerator, wood preservative	Thyroid hormone disruption

margin for the proposed TDI was 100 [17]. However, further evaluation by EFSA became necessary due to new, but not yet evaluated results suggesting that BPA affects neuro-development after *in utero* exposure of experimental animals followed by the exposure during infancy through the milk of mothers exposed to this compound [51]. Moreover, new toxicokinetics studies, including transplacental transport, have demonstrated the need for renewing risk assessment regarding perinatal exposure.

From numerous *in vitro* and *in vivo* studies of the effects on receptors, hormones, the immune system, cell proliferation, apoptosis, intercellular communication, changes in proteomics, genomics (including epigenetic changes), EFSA evaluated their impact on the endocrine system and confirmed that currently used safety margins are still adequate [18].

Exposure modelling and biological monitoring data has allowed EFSA to demonstrate that foodstuffs are a major source of BPA in all population groups [20]. However, the modelling based estimates were considerably lower than those presented in EFSA's opinion issued in 2006. In the previous assessments, high exposure in toddlers was up to 300 ng/kg bw and in 3 month old infants this reached 11 000 ng/kg bw. According to current assessments, the exposure in toddlers was now 857 ng/kg bw and 495 ng/kg bw in infants 3-5 days old. For this opinion, EFSA drew attention that the fact that thermal paper used in printers and cash registers may also be regarded as second great source of BPA in populations older than three years [5]. It was further concluded, that biomonitoring of BPA in urine provides a reliable estimation of the overall exposure from all sources, opening promising perspectives for large scale monitoring programs.

Phthalates

These are mainly used as plasticizers in FCMs made of plastic to increase their flexibility, transparency and durability. Butyl benzyl phthalate (BBP) is used as a plasticizer for polyvinyl and cellulose resins and organic intermediates. Di-n-butyl phthalate (DBP) is used in paper coatings, elastomers and printing inks. Di-ethylhexylphthalate (DEHP) is used as a plasticizer for polyvinyl chloride, especially in manufacturing medical devices and a plasticizer for resins and elastomers. *Lamb* et al. [30] observed adverse effects of DEHP on fertility in experimental mice and proposed a NOAEL for reproduction to be set at a level of 20 mg/kg bw/day. However another study [1], following 60 days of feeding, proposed a NOAEL of 69 mg/kg bw/day for the effects on the gonads and endocrine system. A drastically decreased fertility index was also found in these studies. Other studies have confirmed a profound anti-androgen potential of DEHP [24, 39]. All this data unequivocally indicates that the animal results may trigger concerns when related to humans. Moreover, according to the EFSA's scientific opinion, even if the DEHP dietary intake is below the TDI, there are other sources of this compound that contribute towards the total exposure [15].

Benzophenone

This is used as an additive (photo-initiator UV) for printing inks and may be transferred from the food packaging made of cardboard into the packed foodstuff. Toxicological studies aimed at the hormone-mimetic potential of benzophenone and its derivatives are not unequivocal [37]. Nevertheless, its oestrogenic potential was confirmed in proliferation tests on MCF7 cells [34, 40]. It was also found that benzophenone-1 almost entirely blocked the activity of the 17β-hydroxysteroid dehydrogenase enzyme which is responsible for the testosterone synthesis in *Leidyg* cells. These results suggest that benzophenone may influence gonadal development in experimental animals [42].

In an evaluation of potential health threats arising from benzophenone in some food, *Muncke* [37] stressed that the presence of this compound was confirmed in the foodstuffs packed in multilayer cardboard packaging and that the current TDI, as proposed by the European Union, is 0.01 mg/kg bw. The migration limit into the FCMs was set at 0.6 mg/kg of the foodstuff, assuming that the consumption of packed food by an average adult weighting 60 kg will not exceed 1 kg per day.

The above examples however do not exhaust the issue of endocrine disrupting chemical substances in FCMs. Moreover, the FCMs consist only a small fraction of the numerous sources of human exposure to EDCs. Taking this into consideration, the US Environmental Protection Agency proposed a holistic and systematic approach announcing a two-tiered screening and testing process, where Tier 1 is to identify chemical substances that have potential to interact with the hormone system, and Tier 2 is to establish a quantitative dose-response relationship for adverse effects resulting from toxicological endocrine related outcomes [55]. The EU strategy for endocrine disruptors [21] includes compiling a candidate list of potential endocrine disruptors. The list prioritises the substances that must be evaluated further for any endocrine disrupting effects. Category 1 for potential ECDs contains 194 substances with comprehensive evidence of endocrine-disrupting effects in live animals. The substances should therefore be prioritised for further evaluation of endocrine disrupting properties.

CONCLUSIONS

Several aspects of risk assessment are important when drawing conclusions from toxicological studies

on ECDs released from FCMs into foodstuffs. Above all, consumer safety must be taken into account and that the exposure usually takes place from different routes of exposure and concerns more than just a single substance having endocrine disrupting potential. In the cases where some of them induce similar effects, the possibility of cumulative toxicity should be considered [29]. Furthermore, recommendations on acceptable intake of particular ECDs refer to the average consumer and may not adequately secure those individuals who consume food products containing abnormal amounts of these substances [38]. All these aspects justify a precautionary principle that is applied for making risk management decisions on ECDs in foodstuffs.

Reducing the uncertainty resulting through actual exposure to ECDs from numerous sources and further improvements in developing exposure estimates should be recommended in order to determine the contributing share of FCMs in overall exposures.

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HEALTH OUTCOMES OF VITAMIN D. PART I. CHARACTERISTICS AND CLASSIC ROLE

Julia Wranicz[1], Dorota Szostak-Węgierek[2]*

[1] Chair of Dietetics, Department of Nutritional Physiology, Faculty of Human Nutrition and Consumer Sciences, Warsaw University of Life Sciences, Warsaw, Poland
[2] Department of Human Nutrition, Faculty of Health Sciences, Medical University of Warsaw, Warsaw, Poland

ABSTRACT

Vitamin D is a compound responsible for maintaining mineral homeostasis. It protects against calcium and phosphate deficiency through the effects on the intestine, kidney, parathyroid gland and bone. All mechanisms that help maintain mineral homeostasis of the body are regulated by the vitamin D hormonal form - calcitriol. Synthesis of vitamin D starts in the skin as a non-enzymatic process, which begins during exposure to sunlight, when the absorption of ultraviolet B (UVB) radiation results in convertion of 7-dehydrocholesterol, a metabolite of cholesterol that is stored in the skin, to precholecalciferol (previtamin-D_3) that is immediately converted into cholecalciferol (vitamin D_3). After the skin synthesis cholecalciferol is transported to the liver where it undergoes hydroxylation, what results in formation of calcidiol (25(OH) D_3). The second metabolic process takes place in the kidney, where calcidiol undergoes hydroxylation at the C-1 position to the hormonal, the most active metabolite - 1,25-dihydroxyvitamin D (calcitriol). Vitamin D deficiency may result in bone diseases, such as rickets in children and osteomalacia and osteoporosis in adults. Symptoms of osteomalacia affect mainly the skeletal system and are similar to that observed in rickets. It concerns thoracic kyphosis, pelvis deformities and also the varus knee. Osteoporosis is another condition that is related to abnormalities of mineral homeostasis. It is characterized by the progressive loss of bone mass, impaired bone microarchitecture, and consequently increased fragility and susceptibility to fracture. For the last several years other, non-classic actions of vitamin D_3 have been discussed. It was engendered by the discovery of vitamin D_3 receptor (VDR) in the most of body tissues and cells. Hence, there are many hypotheses which suggest the inverse relationship between vitamin D status and various diseases, such as cancer, autoimmune diseases, diabetes mellitus and others.

Key words: *vitamin D, vitamin D_3, cholecalciferol, mineral homeostasis, vitamin D deficiency, vitamin D_3 receptor, VDR, pleiotropic actions*

STRESZCZENIE

Witamina D jest związkiem chemicznym odpowiedzialnym za utrzymanie homeostazy mineralnej organizmu. Poprzez wpływ na jelita, nerki, przytarczyce i kości zapobiega niedoborowi wapnia i fosforanów. Aktywną formą witaminy D_3 o właściwościach hormonalnych jest kalcytriol, który odpowiada za utrzymanie homeostazy mineralnej organizmu. Pierwszy etap syntezy witaminy D zachodzi w skórze, pod wpływem ekspozycji na światło słoneczne (UVB). Polega on na nieenzymatycznej przemianie 7-dehydrocholesterolu do prowitaminy D (pre-D_3), która natychmiast ulega przekształceniu do cholekalcyferolu (witaminy D_3). Wyprodukowana w skórze witamina D_3 jest następnie transportowana do wątroby, gdzie ulega hydroksylacji, w wyniku której powstaje kalcydiol (25(OH)D_3). Drugi proces metaboliczny zachodzi w nerkach, gdzie kalcydiol ulega hydroksylacji w pozycji C-1 do hormonalnie najbardziej aktywnego metabolitu witaminy D - 1,25-dihydroksywitaminy D (kalcytriol). Niedobór witaminy D może prowadzić do chorób kości, takich jak krzywica u dzieci oraz osteomalacja i osteoporoza u osób dorosłych. Objawy osteomalacji dotyczą głównie układu kostnego i są zbliżone do tych obserwowanych w krzywicy. Są to m.in.: kifoza piersiowa, deformacje miednicy i szpotawość kolan. Osteoporoza to choroba, która także związane jest z zaburzeniem homeostazy mineralnej. Charakteryzuje się postępującą utratą masy kostnej, uszkodzeniami mikroarchitektury kości, i w konsekwencji zwiększoną ich kruchością i podatnością na złamania. Na przestrzeni ostatnich lat pojawiły się doniesienia dotyczące innych, nieklasycznych działań witaminy D_3. Stwierdzono obecność receptora witaminy D (VDR) w wielu tkankach organizmu, które nie biorą udziału w utrzymaniu homeostazy wapniowo-fosforanowej. Świadczy to o wielokierunkowym działaniu tego związku. Pojawiło się wiele hipotez sugerujących związek między niedoborem witaminy D a występowaniem wielu różnych chorób, takich jak: nowotwory, choroby autoimmunologiczne, cukrzyca i inne.

*** Corresponding author:** Julia Wranicz, Chair of Dietetics, Department of Nutritional Physiology, Faculty of Human Nutrition and Consumer Sciences, Warsaw University of Life Sciences – SGGW, Nowoursynowska street 159c, 02-776 Warsaw, Poland, phone +48 22 59 37 030, fax +48 22 59 37 031, e-mail: julia_wranicz@sggw.pl

Słowa kluczowe: *witamina D, gospodarka mineralna, niedobór witaminy D, witamina D_3, cholekalcyferol, receptor witaminy D_3, działanie plejotropowe*

INTRODUCTION

Vitamin D is a compound responsible for maintaining mineral homeostasis. It regulates serum calcium and phosphorus concentrations by affecting their metabolism and absorption. Vitamin D acts mainly in the kidneys, the intestine and the bone by its hormonal form calcitriol ($1,25(OH)_2D_3$). Vitamin D deficiency may result in bone diseases, such as rickets in children and osteomalacia and osteoporosis in adults [10, 21].

However, for the last several years other, non-classic actions of vitamin D_3 have been discussed. It was engendered by the discovery of vitamin D_3 receptor in the most of body tissues and cells. Hence, there are many hypotheses which suggest the inverse relationship between vitamin D status and various diseases, such as cancer, autoimmune diseases, diabetes mellitus and others [1, 5]. Researchers point out that calcitriol, an active metabolite of vitamin D, affects gene activity. It binds to the nuclear VDR receptor what results in its activation and regulation of 5% of human genome activity. It indicates effectiveness and pleiotropic actions of vitamin D_3 [16].

Vitamin D_3 (cholecalciferol) is formed in the skin from 7-dehydrocholesterol that is present in plasma membranes of the epidermis and dermis, under the influence of UVB rays [11]. The effectiveness of skin synthesis depends on insolation and varies by region and season. In Poland efficient irradiation occurs from April to September, between 10 am and 3 pm. It is recommended to expose 18% of the skin surface of the body for at least 15 minutes daily, without the use of protective UV filters. Skin synthesis is the major vitamin D source. Other sources are food products, such as oily fish and cod liver oil [3]. Vitamin D can be ingested also through supplements and fortified foods.

CHARACTERISTICS OF VITAMIN D

Vitamin D is a generic term for the group of chemical compounds of molecular steroid structure. The most valuable are ergocalciferol-vitamin D_2, found in plants and fungi, and cholecalciferol-vitamin D_3, present in animal products [20]. Vitamins D_2 and D_3 do not reveal biological activity. In humans ergocalciferol and cholecalciferol are transformed to 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$,) - calcitriol, recognized as the most active form of vitamin D [11, 21].

Vitamin D, discovered by *Mc Collum* et al. in 1918, differs from other vitamins. The human body can produce it in sufficient amount from its provitamin that is present in the skin. In addition, vitamin D is characterized by hormonal activity, what means that its active metabolites are synthesized in the kidney and in the liver and transported by the blood to various target tissues and organs, such as intestinal epithelium and bones [17].

Skin synthesis of vitamin D is a non-enzymatic process, which begins during exposure to sunlight, when the absorption of ultraviolet B (UVB) radiation results in conversion of 7-dehydrocholesterol, a metabolite of cholesterol that is stored in the skin, to precholecalciferol (previtamin-D_3). Precholecalciferol is inherently unstable and is immediately converted into cholecalciferol - vitamin D_3, under the influence of body temperature, and then is absorbed into the blood stream [15]. Apart from skin synthesis, cholecalciferol can be supplied by the diet.

After the skin synthesis of vitamin D_3, its metabolic processes are continued in the internal organs. Cholecalciferol is transported from the skin to the liver by the blood. In hepatic cells it undergoes hydroxylation, what results in formation of 25-hydroxyvitamin D, that is called calcidiol ($25(OH)D_3$). After getting into the blood, calcidiol binds to the specific transport protein - gc-globulin (DBP). The $25(OH)D_3$-DBP complex is the transport form of vitamin D [22]. The second metabolic process takes place in the kidney, where calcidiol undergoes hydroxylation at the C-1 position to the hormonal, the most active metabolite - 1,25-dihydroxyvitamin D (calcitriol), and also hydroxylation at C-24 position to the probably inactive metabolite 24,25-dihydroxyvitamin D (24 -hydroxycalcidiol) [4, 15].

On the contrary to the non-enzymatic synthesis of vitamin D in the skin, its metabolism in the liver and kidney requires enzymes – hydroxylases. Their activity depends on many factors. It was found that the liver 25-hydroxylase activity increases with the amount of substrate, concentration of vitamin D-binding protein and certain drugs (e.g. antiepileptic). Its activity is decreased by the final vitamin D metabolites. Activities of the enzymes that catalyze 1-α and 24-hydroxylation of calcidiol depend on the serum concentrations of calcium and phosphate, and also some hormones and prostaglandins [17, 21].

The strongest stimulators of calcitriol synthesis are: parathyroid hormone (PTH), parathyroid hormone-related protein (PTHrP), hypocalcemia and hypophosphatemia. Because of the feedback system, increased calcitriol level exerts the inhibitory effect on its own formation. Other factors that inhibit the synthesis of calcitriol are: deficiency of parathyroid hormone and

PTHrP, hypercalcemia, hyperphosphatemia and calcitonin [17, 21].

Concentration of vitamin D metabolites in the blood is determined not only by their synthesis, but also by the process of catabolism and excretion. There are three major metabolites of vitamin D present in the serum: $25(OH)D_3$, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$, which are regularly eliminated from the body by conversion to more polar compounds in the target tissues and their excretion in bile, faeces and urine. Calcidiol, the hepatic metabolite, is eliminated by the 1- and 24-hydroxylation and excreted in bile, after its combination with glucuronic or sulfuric acid [17]. Other metabolites of vitamin D are also combined with glucuronic or sulfuric acid in the liver, excreted in bile into the intestine, and pass into the enterohepatic recirculation. It was found that physiologically only 3% of vitamin D metabolites circulating in the blood are excreted in urine and faeces. The daily excretion of vitamin D in humans is 1-7μg, mainly in the faeces, with the aid of bile salts. Small amounts appear also in urine. Human resources of vitamin D are stored mainly in the liver and adipose tissue [4, 17].

ROLE IN MINERAL HOMEOSTASIS – CLASSIC FUNCTION OF VITAMIN D

Vitamin D is the major regulator of calcium and phosphate homeostasis. It protects against calcium and phosphate deficiency through the effects on the intestine, kidney, parathyroid gland and bone. All mechanisms that help maintain mineral homeostasis of the body are regulated by vitamin D hormonal form - calcitriol [21]. Calcitriol acts in the gut affecting the intestinal absorption of calcium and phosphate. It increases calcium entry into enterocytes and accelerates calcium flux through the cell cytosol. Calcitriol enhances calcium transfer through the basement membrane of the intestinal epithelium into the circulatory system. In the phosphate transport through the intestinal mucous vitamin D-dependent sodium-phosphate co-transporter is involved.

Vitamin D is essential for proper bone mineralization. This is carried out by maintaining appropriate level of calcium and phosphate in the blood. Besides, calcitriol can stimulate production of collagen by osteoblasts [15].

In the mineral homeostasis $1,25(OH)_2D_3$ works in concert with parathyroid hormone to exert its beneficial effects on the plasma levels of ionised calcium and phosphate. The physiologic cycle starts in parathyroid gland where calcium is sensed by the calcium receptor. When the level of calcium in plasma decreases, parathyroid hormone is secreted and stimulates the renal enzyme 25(OH)D-1-aa-hydroxylase to produce more $1,25(OH)_2D$ from 25(OH)D. Parathyroid hormone, which stimulates the production of calcitriol in the kidney, also increases renal and intestinal reabsorption of calcium, whereas the phosphate transport is inhibited [6]. The resulting rise in $1,25(OH)_2D$ causes a boost in calcium transport within the intestine, bone, and kidney.

Calcitriol, $1,25(OH)_2D$, acts together with parathyroid hormone to mobilize calcium from bone tissue by osteoclastic stimulation. In the case of deficiency of the active form of vitamin D, resistance to the action of parathyroid hormone on the bone occurs [19]. Additionally, calcitriol works like a hormone in the intestinal tract where its activity is related to the interaction with VDR. Inactive calcitriol receptor in intestinal epithelial cells is located in the cytoplasm. After binding $1,25(OH)_2D$, the calcitriol-receptor complex translocates to the nucleus. The ligand-receptor complex acts like a transcription factor by promoting the expression of the gene encoding calcium binding protein. As a result, levels of calcium binding protein increase what enables the cells to transport more calcium (Ca^{2+}) from the intestine [20].

As a result of the feedback system high calcium plasma level achieved by the action of parathyroid hormone and calcitriol returns to normal. It is sensed by calcium receptors of the parathyroid gland, which regulate PTH secretion. Inhibition of PTH secretion depends not only on the feedback action of calcium, but also on the feedback loop involving $1,25(OH)_2D$ that directly suppresses PTH synthesis in the parathyroid gland [7].

CLASSIC DISORDERS RELATED TO VITAMIN D DEFICIENCY AND OVERDOSE

The most common diseases resulting from vitamin D deficiency, are: rickets that occurs in children and osteomalacia and osteoporosis that affect adult population [12, 22, 23]. In rickets severe lack of vitamin D_3 is followed by inhibition of calcium absorption in the intestine, excessive phosphate and calcium excretion in the kidney, and consequently decrease in the serum calcium concentration. In the aim to maintain proper calcium level parathyroid hormone is secreted. It stimulates release of calcium from the bones. This process results in the bone decalcification and subsequently symptoms of active rickets [22].

Rickets occurs in young children, and its first characteristic symptom is occipital malacia. Softening of the skull bones is usually recognized during palpation. In consequence of malacia, the occiput becomes flattened and the skull bones form a square shape with enlarged circumference [14, 22]. In children who start to walk varus or valgus deformities of the knees and also pelvis defects may occur. They may be accompanied by skeletal muscle flaccidity, which results in a delay in motor development, increase in abdominal circumference,

abdominal distension and constipation. The described above early symptoms usually disappear in the later life. Late symptoms which remain in adulthood are: the flat feet, defects of the spine and chest construction [22]. Tetany is another rickets symptom. It results from hypocalcaemia and is characterized by excessive electrical and mechanical excitability of the neuromuscular system and is followed by tonic cramps [14].

The initial phase of rickets is characterized by the decreased serum concentration of the vitamin D_3 metabolite ($25(OH)D_3$), increased alkaline phosphatase activity, followed by the increased excretion of phosphate in urine. Additionally, in the advanced rickets a decreased level of serum phosphorus is observed. Calcium concentration is usually in the lower normal range. Prevention of rickets is based on the exposition to ultraviolet rays, and application of vitamin D_3 preparations [14].

Osteomalacia is a disease similar to rickets, but affects the adult population. It is caused by the deficiency or absence of vitamin D_3 in the body, what results in impaired absorption of calcium and phosphate ions in the intestine, and subsequently in their reduced resources in the body. It is followed by reduction of the bone mineral density, which makes them less resistant to mechanical damage and fracture. Apart from the vitamin D deficiency, also other factors, such as use of antiepileptic drugs, consumption of large amounts of alcohol and hepatic cirrhosis, may contribute to osteomalacia [12, 18, 23].

Symptoms of osteomalacia affect mainly the skeletal system and are similar to that observed in rickets. It concerns thoracic kyphosis, pelvis deformities and also the varus knee. Other characteristic symptoms are: the disturbance of gait called "the duck walk", and muscle weakness, which results in the rapid fatigue [18, 23]. Osteomalacia therapy is based on the oral supplementation of vitamin D_3 and calcium, as well as administration of phosphates. Exposure to UV radiation, which stimulates the cutaneous synthesis of vitamin D_3 is also recommended [23].

Osteoporosis is another condition that is related to abnormalities of mineral homeostasis. It is characterized by the progressive loss of bone mass, impaired bone microarchitecture, and consequently increased fragility and susceptibility to fracture [12, 13]. Among factors that contribute to this disease there is the deficiency of vitamin D in the body. The beginning of the disease is usually asymptomatic. The most common clinical signs, that develop in advanced cases of osteoporosis, include loss of height due to vertebral compression fractures, back pain and the formation of senile i.e. excessive thoracic kyphosis. The disease is often complicated by bone fractures, that occur even at minor injuries, such as the so-called falls from "the own height" [13].

The increased risk of fractures in osteoporosis due to deficiency of vitamin D is also related to its effect on the function of skeletal muscles. The vitamin D active metabolite - $1,25(OH)_2D_3$ binds to the VDR nuclear receptor in muscle cells and thus stimulates biosynthesis of proteins that are responsible for the growth of these cells, what results in the increase in muscle strength. Therefore, it is postulated that vitamin D deficiency leads to weakening of the antigravity muscles, grip strength and also impairment of physical endurance of patients. Apart from pharmacological treatment of osteoporosis, it is important to comply with a well-balanced diet, rich in calcium, vitamin D and protein [13].

Another problem is the vitamin D hypervitaminosis. It is unlikely to be caused by the diet or intense exposure to ultraviolet radiation. However, during the treatment of certain diseases, such as sarcoidosis, tuberculosis, or idiopathic hypercalcemia, therapeutic doses of vitamin D may cause symptoms of poisoning. The 25-hydroxyvitamin concetrations in the blood of more than 200 ng/mL (500 nmol/L) are considered to be potentially toxic and may lead to hypercalcemia and hyperphosphatemia. Among symptoms of hypercalcemia there are: nausea and vomiting, loss of appetite, constipation, weakness, fatigue, excessive thirst, increased urination, itching and headaches. Besides, hypercalciuria may result in formation of calcium deposits in tissues and organs, and also calcification in the kidneys and hence the failure of this organ [8, 9].

PLEIOTROPIC ACTION OF VITAMIN D

Development of molecular biology and diagnostic methods enabled the more detailed research of functions of vitamin D in the human body. A number of new types of its activities were identified. It was even proposed that proper vitamin D supplementation is rather an endocrinologic than nutritional issue. Calcitriol binds to the nuclear VDR receptor, what results in control of 500 gens. This indicates pleiotropic actions of vitamin D_3. Apart from the tissues and organs responsible for maintaining calcium and phosphate homeostasis, the

Table 1. Cells, tissues and organs with the vitamin D_3 receptor (VDR) [16]

Cells, tissues and organs with the VDR		
Adipose tissue	Skin tissue	Placenta
Osseous tissue	Hair follicle	Uterus
Cartilaginous tissue	Kidney	Ovary
Smooth muscles	Fetal liver	Testicle
Cardiac muscle	Lungs	Epididymis
Fetal muscle tissue	Brain	Parotid gland
Adrenal gland	Parathyroid gland	Retina
Cancer cells	Pituitary gland	Bone marrow
Stomach	Thymus gland	Pancreatic β-cells
Small intestine	Thyroid gland	Osteoblasts
Large intestine	Mammary gland	Lymphocytes B and T

vitamin D receptor was identified in 36 other sorts of tissues and cells in the human organism (Table 1.), e.g. in the cardiac muscle, smooth muscles, brain, endocrine glands and lymphocytes B and T.

In addition, 1-α-hydroxylase, the enzyme that converts non-active $25(OH)D_3$ to the active form $1,25(OH)_2D_3$, was found in many various localizations, apart from the kidney (Table 2). For example, 1α-hydroxylase activity was observed in endothelial cells, smooth muscle cells of the blood vessels, and also activated macrophages. This type of the enzyme is called a peripheral hydroxylase. Its importance for the vitamin D effect in the human body is increasingly distinguished.

Table 2. Presence and activity of the peripheral 1α-hydroxylase of 25-hydroxyvitamin D [16]

Site	mRNA	Protein	1-α-hydroxylase activity
Large intestine	+	+	+
Dendritic cells	+	-	-
Endothelial cells	+	+	+
Brain	-	+	-
Mammary gland	+	+	+
Pancreatic islands	+	+	+
Parathyroid gland	+	+	+
Placenta	+	+	+
Prostate gland	+	+	+
Skin (keratinocytes)	+	+	+
Macrophages (activated)	+		+

Discovery of the vitamin D pleiotropic activity in the majority of body cells and tissues stemmed from numerous epidemiological surveys that revealed correlation between low vitamin D status and increased risk of diseases of various etiologies, including autoimmune and cardiovascular diseases, cancers, diabetes and also infectious diseases [2].

The role of vitamin D in the pathogenesis of these diseases will be described in the second part of this paper.

SUMMARY

The classic role of vitamin D is maintaining mineral homeostasis. Its deficiency may result in rickets, osteomalacia and osteoporosis. Except of the bone, intestine and kidney, vitamin D also acts in other tissues and organs. Further studies addressing the mechanisms by which vitamin D affects and the proper supplementation required are needed.

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DETERMINATION OF CHLORAMPHENICOL IN MILK POWDER USING LIQUID-LIQUID CARTRIDGE EXTRACTION (CHEM ELUT) AND LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

*Iwona Zawadzka, Lech Rodziewicz**

Laboratory of Chemical Examination in Foodstuffs, Department of Hygiene Veterinary,
Voivodeship Veterinary Inspectorate, Białystok, Poland

ABSTRACT

Background. The European Union prohibits the use of chloramphenicol (CAP) as a veterinary drug in food-producing animals. Nevertheless, CAP have been detected in milk products (liquid milk and milk powder). Therefore, it is necessary to develop sensitive methods for determining CAP residues in milk powder.

Objective. The aim of this study was to develop and validate a confirmatory method for determination of CAP in milk powder.

Material and methods. Chloramphenicol was determined in milk powder using LC-ESI-MS/MS in negative mode. After fat removing milk powder sample was extracted/cleaned-up with a Chem Elut extraction cartridge. Separation was achieved on a Phenomenex Luna C-18 column with acetonitrile-water as a mobile phase. The mass spectrometer was operated in multiple reaction monitoring mode (MRM). Four transitions were monitored m/z 321→152, 321→194, 321→257 (CAP) and 326→157 (IS CAP-d5).

Results. Linearity, accuracy, precision, decision limit (CCα), detection capability (CCβ) and ruggedness were determined for m/z 321→152. The mean relative recoveries (inter standard-corrected) of CAP from whole milk powder spiked at levels 0.1, 0.2, 0.3 and 0.6 μg/kg were in the range 95 - 103%. Relative standard deviation (RSD%) of recoveries at all spiked levels were less than 14%. RSDs within-laboratory reproducibility calculated at fortification of 0.3 μg/kg was less than 16%. CCα and CCβ were below 0.1 μg/kg.

Conclusions. The developed LC-MS/MS method allows the determination of CAP in milk powder. The method was validated according to the Commission Decision No. 2002/657/EC requirements. This method can be applied to determination CAP in whole and skim milk powder.

Key words: *chloramphenicol, veterinary drug residues, milk powder, Chem Elut; LC-ESI-MS/MS*

STRESZCZENIE

Wprowadzenie. Unia Europejska zabroniła stosowania chloramfenikolu (CAP) jako leku weterynaryjnego u zwierząt, których produkty są przeznaczone do spożycia. Pomimo tego, CAP jest wykrywany w produktach mleczarskich (mleko i mleko w proszku).

Cel. Celem badań było opracowanie i zwalidowanie metody pozwalającej na oznaczanie CAP w mleku w proszku.

Materiał i metoda. CAP był oznaczany w mleku w proszku metodą LC-ESI-MS/MS w trybie jonizacji ujemnej. Po usunięciu tłuszczu próbka była ekstrahowana/oczyszczana za pomocą ekstrakcyjnych kolumienek Chem Elut. Do rozdziału CAP stosowano kolumnę chromatograficzną Phenomenex Luna C-18. Fazę ruchomą stanowił acetonitryl/woda. Spektrometr masowy pracował w trybie monitorowania wybranych reakcji (MRM). Cztery przejścia były monitorowane m/z 321→152, 321→194, 321→257 (CAP) i 326→157 (IS CAP-d5).

Wyniki. Liniowość, odzysk, precyzja, limit decyzyjny (CCα), zdolność wykrywania (CCβ) i odporność metody zostały wyznaczone dla 321→152. Średni względny odzysk CAP z mleka pełnego był wyznaczony dla próbek wzbogaconych na poziomach odpowiadających 0.1, 0.2, 0.3 i 0.6 μg/kg i mieściły on się w zakresie 95 - 103%. Względne odchylenie stadardowe (RSD%) dla wszystkich poziomów było mniejsze niż 14%. Powtarzalność wewnątrzlaboratoryjna (RSDs) obliczona dla poziomu wzbogacenia 0.3 μg/kg była mniejsza niż 16%. CCα and CCβ były poniżej 0.1 μg/kg.

Wnioski. Opracowana metoda LC-ESI-Ms/MS pozwala oznaczyć CAP w mleku w proszku. Metoda została zwalidowana zgodnie z wymaganiami decyzji Komisji nr 2002/657/WE. Metoda może być stosowana do mleka pełnego i odtłuszczonego.

Słowa kluczowe: *chloramfenikol, pozostałości leków weterynaryjnych, mleko w proszku, Chem Elut, LC-ESI/MS/MS*

*** Corresponding author:** Lech Rodziewicz, Laboratory of Chemical Examination in Foodstuffs, Department of Hygiene Veterinary, Voivodeship Veterinary Inspectorate, Białystok, Poland, phone +48 85 6510 229
e-mail: rodziewicz@wiw.bianet.com.pl

INTRODUCTION

Milk is an important food in human nutrition because it contains essential constituents, such as proteins with high biological quality, carbohydrates useful for the nervous systems development, and essential fatty acids, vitamins and minerals. Milk powder can be contaminated with chloramphenicol (CAP). Human exposure to CAP could cause Grey syndrome, bone marrow depression and fatal aplastic anemia. Consequently, its use in food-producing animals and animal feed products is prohibited in European Union (EU). As a prohibited substance, zero tolerance applies. Currently, CAP has Annex IV classification under EU legislation [1]. No maximum residue limit (MRL) has been established for this antibiotic. Thus, the EU has assigned a minimum required performance limit (MRPL) for CAP in food of animal origin at a level of 0.3 μg/kg [2]. However, many companies introduced their own action limits forcing the development of sensitive assays able to detect 0.1 μg/kg.

A variety of method have been used to determine CAP in food of animal origin. Nowadays, as a screening test for animal-derived food, enzyme-linked immunosorbent assay (ELISA) method is used. ELISA method has a detection limit similar to that of liquid chromatography – tandem mass spectrometry (LC-MS/MS). However, commonly occurring problems with the ELISA method is one of false positive results [3, 17] Common confirmatory methods for the determination of CAP in animal food have been based on LC-MS/MS. Several LC-MS/MS methods have been developed for the determination of CAP residues in animal tissues [11], honey [12] liquid milk [8] and milk powder [9, 10, 13].

A detection of trace levels of CAP residues in rich matrix such as milk powder requires efficient sample preparation procedure (extraction and clean-up). Sample preparation procedures such as liquid-liquid extraction (LLE) [10] and solid-phase extraction (SPE) are common for extraction and clean-up. However, due to its ease of operation and environmental interest SPE has gained bigger popularity than LLE. Sorbent including C-18 [9, 16] Oasis HLB [3] and molecular imprinted polymer – MIP [7, 13] cartridges were successfully applied for the analysis of CAP in milk powder.

A potential drawback of SPE procedure is that it requires a large number of individual steps (column conditioning, sample application, washing and elution). In order to shorten the sample clean-up procedure Chem Elut extraction cartridge was applied. Chem Elut cartridge with diatomaceous earth instead of classical SPE methods was used to extract and purify the samples. The principle of SPE using diatomaceous earth is closely related to conventional liquid-liquid extraction. It involves the absorption of the aqueous phase on the diatomaceous earth, a porous material that acts as a support for the aqueous phase. This provides a large surface area for partition into an eluting solvent, which flows through the immobilized specimen under gravity, eluting the analytes of interest. However, large volumes of hazardous organic solvents are required. SPE with diatomaceous earth cartridges was previously applied for the determination of pesticide residues in fruit, vegetable samples and drugs in body fluids. Diatomaceous earth (Chem Elut, Extrelut) was also applied for the analyses of CAP by LC-MS-MS in urine [14, 15], plasma [15], shrimp tissue [4], liquid milk [8] and honey [6].

The aim of this work was to develop and validate LC-MS/MS method for determination and confirmation of the chloramphenicol residues in milk powder.

MATERIAL AND METHODS

Chemicals and reagents

Ultrapure water was obtained from Milli-Q system Millipore (Bedford, MA, USA). Acetonitrile LC-MS grade, ethyl acetate LC grade and hexane LC grade. Chem Elut extraction cartridges (5 ml, unbuffered) were provided from Varian (Part number 12198006, The Netherlands).

Chloramphenicol (CAP) was purchased from Sigma-Aldrich (Schnelldorf, Germany). Internal standard deuterated chloramphenicol d5 (100 μg/mL in acetonitrile) was purchased from Cambridge Isotope Laboratories (FSD-117-100, 98%).

Standard solution

Individual stock solutions of CAP at 1 mg/ml was prepared in acetonitrile. This solution was diluted in acetonitrile to prepare an intermediate standard solution of 20 μg/ml. Working solution of 5.0 ng/ml was made by diluting intermediate standard with acetonitrile. Internal standard of CAP-d5 was prepared by dissolving the ampoule with 100 μg/ml in acetonitrile, which was adequately diluted to obtain a working solution of 3.0 μg/ml (IS). The stock standards solution (1.0 mg/ml) kept at –20°C were stable for 1 year. The intermediate standard solution (20 μg/ml) stored at –20°C was stable 3 month, while the working standard solution (5.0 ng/ml) stored at 4°C was stable for 3 months.

Samples preparation

The milk powder samples were collected by the Polish Veterinary Inspectorate at inspection points. Samples were stored at +4°C until analysis.

A 5.0 ± 0.05 g of whole milk powder (26-28% fat) was weighed into a 100 ml plastic centrifuge tube and working solution (IS) was added (3 ng/ml CAP-d, 500 μl). Subsequently, 50 ml of water was added and

the tube was capped tightly. The mixture was placed in water bath at 40 °C and mixed about 10 min until a homogeneous ample was obtain. After cooling down to room temperature the mixture was placed in the freezer at temperature below –20°C for at least 10 min. Precipitated fat was removed by centrifuging at 4000 x *g* for 10 min at –4°C. After centrifugation supernatant was filtered through folded filters. The 5 ml sample was applied to 10 ml Chem Elut cartridge and left for 5 min. CAP was eluted from the cartridge in two stages with 10 ml and 8 ml of ethyl acetate. The ethyl acetate was evaporated to dryness under a stream nitrogen using a heating block at 45°C. The dry residue was dissolved in 1 ml of acetonitrile and 2x1 ml of hexane were added. The sample was mixed and hexane phase was discarded. Next, sample was evaporated once again to dryness under stream of nitrogen using a heating block at 45°C and residue was dissolved in acetonitrile/water (20:80, v/v, 250 µl) and filtered trough 0.45 µm PVDF filter into amber vial. An aliquot of 10 µl was injected into LC-ESI-MS-MS.

Matrix matched calibration curve

The milk powder matrix matched calibration curve was prepared and used for quantification. The calibration curve was built by spiking blank matrix samples of milk powder with CAP (levels from 0 to 0.6 µg/kg, five points). A fixed amount of internal standard was added to all samples (3 ng/ml CAP-d5, 500 µl). The equation was $y=a + bx$, where x was the injected amounts of CAP in µg/kg and y was the peak area ratio (CAP/IS). The calibration curve was obtained relating ratio CAP area m/z 321→152/326→157 (IS) with CAP concentration in µg/kg. Peak area (analyte to internal standard) ratios were calculated using Analyst 1.5.1 Software.

LC-ESI-MS/MS analysis

All analyses were performed on an Agilent 1200 series liquid chromatography interfaced to an Applied Biosystems/MDS SCIEX Q TRAP 5500 mass spectrometer (Concord, Ontario, Canada). The chromatographic separation was performed in a C18 column (150 mm x 2 mm id., 3 µm) (Phenomenex, Torrance, USA). The LC flow rate was set at 300 µl/min, the injection volume at 10 µl and the column temperature at 40°C. The mobile phase was acetonitrile (A) and water (B). The linear gradient program was: 0.0–0.1 min 0% A; 0.1–3.0 min 80% A; 3–12 min 80% A; 12–12.3 min 0% A; and 12.3–20 min 0% A. The LC flow was directed into the MS detector between 4 and 8 min using a VICI diverter (Valco Instrument Co. Inc., Houston, TX). The mass spectrometer was used in negative mode. The optimized source and gas parameters were as follows: curtain gas (CUR), 15 psi; collision gas (CAD), 7 psi; ion source temperature (TEM), 400°C; ion source gas 1 (GS1), 45 psi; ion source gas 2 (GS2), 60 psi; ion spray voltage, –3500 V and dwell time 150 ms. Nitrogen was used as collision gas. The instrument was operated in Multiple Reaction Monitoring (MRM) mode, using the following transitions: m/z 321→152, 321→194, 321→257 for CAP and m/z 326→157 for CAP-d5; with collision energies (CE) of 18, 14, 14 eV and 18 eV, respectively.

RESULTS AND DISCUSSION

Extraction and clean-up

Sample extraction/clean-up can be done either by applying a vacuum to pull the sample or solvent through the SPE cartridge, or by allowing gravity to pull the sample or solvent through. In our preliminary studies, extraction/clean-up procedure was investigated by using gravity cartridge Chem Elut (Varian) and Extrelut NT 3 (Part number 1.15095.0001, Merck). In this experiment, the whole milk powder samples were spiked CAP at 0.3 µg/kg. The sample was passed through the cartridge. CAP was eluted using of the ethyl acetate (section 'Sample preparation'). The extraction/clean-up was assessed through recoveries. The recoveries were calculated by comparing the area of analytes in speaking matrix to that of standard solution at the same contraction. Chem Elut cartridge and Extrelut cartridge allowed to achieving the recoveries of >90% and > 60%., respectively. The Chem Elut was selected in this work.

Matrix effects

Matrix effects of ion suppressing was checked, which is a common problem of ESI technique. The matrix effects may result as positive or negative responses depending on the level of ion suppression and can greatly affect the method accuracy and reproducibility. The ion suppression effects were evaluated by typical experiment system. The blank whole milk powder sample was prepared as described in section Sample preparation. The dry extract was dissolved in mobile phase. The mobile phase was mixed with CAP solution in mobile phase to the final concentration of 0.1, 0.3 and 0.6 µg/kg. Then, spiked samples were compared to standard in mobile phase (0.1, 0.3 and 0.6 µg/kg). The signals intensities (peak areas) of m/z 321→152 and 326→157 CAP-d5 (IS) transitions were observed. The signals intensities for the CAP were lower than the standard solution. The matrix effects were in the range –29% to –37% for 321→152 and –22% to –28% for 326→157 ("–" represents a loss of analyte signal - ion suppression). These results revealed that determination of CAP was affected by the interferences from real samples to some extent. Therefore, to provide reliable

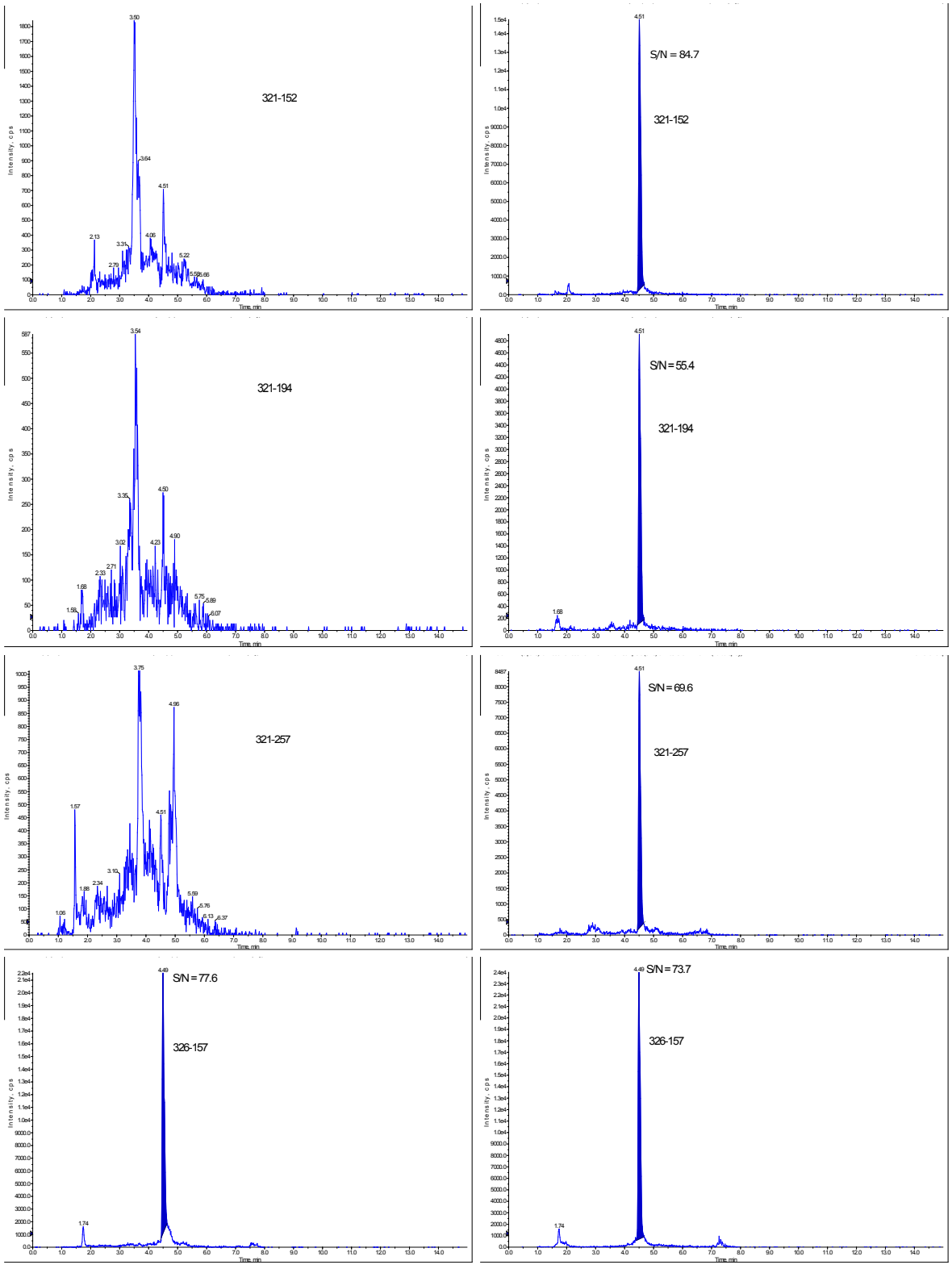

Figure 1 LC-ESI MS/MS chromatograms of blank and spiked whole milk samples at 0.1 µg/kg.

results, matrix-matched calibration curves were chosen the roughout this study.

Identification

The performance of analytical method is evaluated by checking the identification criteria: the signal-to-noise ratio (S/N) of diagnostic ions have to be grater then three, the relative ratio of retention time of the analyte to that of the IS corresponded to that of the calibration solution within a ±2.5% tolerance for liquid chromatography and the relative peak area ratios of the various transition reactions within the tolerances set by the EU criteria [1]. All these were fulfilled with the samples

spiked at 0.1, 0.2, 0.3 and 0.6 μg/kg. At the lowest spiked level (i.e. 0.1 μg/kg), the S/N was greater than 3.

All the chromatograms obtained throughout the validation study showed very good stability of the relative retention time for the spiked samples and for the positive samples with relative deviations always better than ±2.5%. The peak area ratios of the various transition reactions were calculated from the spiked samples. The ratios vary from 30 to 35% for 194/152 and 55 to 69% for 257/152. The mean ratios for 194/152 and 257/152 was 32 (6.1) ±25% (peak are ratio (RSD) ± tolerance given in Decision 2002/657/EC) and 56 (7.9) ±20%, respectively. A similar results were observed for pure standard solution prepared in water. The mean ratios for 194/152 was 31% and for 257/152 was 59%.

Validation

For validation specificity, linearity, accuracy, precision (repeatability, and reproducibility), decision limit (CCα), detection capability (CCβ) and ruggedness were determined according to Commission Decision 2002/657/EC [1].

The specificity of the developed method was evaluated by analyzing 20 different blank and spiked whole milk powder samples at 0.1 μg/kg in order to investigate possible interference retention time. Figure 1 shows a blank sample and a spiked milk powder at 0.1 μg/kg. All blank milk powder extracts exhibited no significant interferences at retention times of target and internal standard. Good chromatographic signals were obtained for CAP and CAP-d5 (IS) in powder milk treated with described method. The typical LC retention time of CAP and CAP-d5 (IS) was around 4.8 min.

The linearity was evaluated by analyzing the calibration curves of spiked whole milk powder in the 0.0–0.4 μg/kg (five calibration points, six curves). These samples were randomly chosen from previously analyzed CAP free samples. The matrix calibration curves were linear in the range 0.0 – 0.6 μg/kg. The correlation coefficient and slope were within the range 0.994 – 0.999 and 3.51 – 3.65 respectively.

The accuracy and precision-repeatability of CAP were measured in blank spiked samples of whole milk powder that were spiked at 0.1, 0.2, 0.3 and 0.6 μg/kg. The samples were analysed on different days close to each other, with the same instrument and same operators. Six replications were obtained for each concentration. Accuracy was assessed through relative recovery. The relative recovery (%) were calculated against matrix matched standard curves using internal standard added before sample preparation. The relative standard deviation (RSD, %) was calculated for each level. The relative recovery and precision (repeatability) date obtained from the analyses of blank spiked samples at four contraction levels are reported in Table 1. The results show good relative recoveries ranged between 95 and 103% with a good RSD, less than 14%. The high relative recoveries and the use of internal standard ensure that the method is suitable for determination of CAP in milk powder at the level required.

Absolute recovery (%) of analytes was determined by comparison of peak areas from blank whole milk powder samples spiked with known amounts of CAP (0.1, 0.2, 0.3 and 0.6 μg/kg) before the preparation procedure to peak area from matrix extract spiked after it The results were summarized in Table 1. The absolute recoveries were in the range of 69 to 77%. The mean absolute recover was 72%.

Table 1. Relative recovery, precision and absolute recovery for whole milk powder (n=6)

Spike levels (μg/kg)	0.1	0.2	0.3	0.6
Relative recovery (%)	103	97	102	95
RSD (%)	12.8	9.0	8.7	13.9
Absolute recovery (%)	71	72	77	69

For an evaluation of reproducibility only within-laboratory reproducibility was considered. The precision within-laboratory reproducibility was calculated in spiked samples at concentration of MRPL (0.3 μg/kg). They were analysed on three different days (3x6), with the same instrument but different operators. The overall RSDs was calculated as within-laboratory reproducibility. The average recovery was 67% with RSDs 15.8%.

To comply with Commission Decision 2002/657/EC [1] the CCα and CCβ were determined. Decision limit (CCα) means the limit at and above which it can be concluded with an error probability of α=0.1% that the sample is non-compliant in case there is a non-zero test limit. Detection capability (CCβ) means the smallest content of the substance that may be detected, identified or quantified in a sample with the error probability of β=95% .

Decision [1] defines two methodologies for determination of CCα and CCβ during method validation. The first method is based on determination of signal to noise (S/N) ratios in blank samples and matrix material spiked at the CCα. The second method refers to the international standard ISO 11843-2 [5]. In this work analytical limits CCα and CCβ were calculated by applying the matrix calibration curve procedure according to case 1 of ISO 11843-2 - constant standard deviation. These method parameters are to be used instead of the familiar limit detection (LOD) and limit of quantification (LOQ). The CCα and CCβ were calculated using two calibration curves (at five levels 0.0, 0.1, 0.2, 0.3 and 0.6 μg/kg) from six different experiments on different whole milk powder matrix and different days. Curves were constructed using analyte/internal standard peak area ratio versus concentration of analyte. CCα and

CCβ were calculated for m/z 321→152/ 326→157 (IS) ion transitions. The mean values CCα and CCβ were 0.026 μg/kg and 0.033 μg/kg. These values were below MRPL of 0.3 μg/kg [2].

The ruggedness was tested by introduction of seven small but deliberate changes in operation parameters (variables) and by the consequent assessment of their influence of the method results. We developed 8 testes in accordance with *Youden* approach [1], using a matrix whole milk powder spiked at 0.3 μg/kg. Seven independent sample preparation parameters examined are reported in Table 2. *Student t*-test at 95% confidence limit was used to compare the high level with varied low level. No significant differences on the performance were observed, indicating good ruggedness of the method.

Table 2. Sample preparation parameters setting applied in the robustness experimental design

Sample preparation parameters	High level	Low level
Water bath temperature (°C)	45	35
Mixing time (min)	15	5
Freezing out time (min)	15	5
Elution mode	after 10 min	immediate
Defating mode	1 x 2 ml	2 x 1 ml
Extract evaporation temperaturę (°C)	50	40
% ACN in final mixture	22	18

Application to skim milk powder

The present procedure was also applied to analyse CAP in skim milk powder, containing about 1-2 % fat. LC-MS/MS was therefore applied, without any modification to whole milk powder treated milk spiked with the CAP at the same contraction levels as the whole milk samples. In all instances, basically the same results were obtained as for whole milk powder for parameters such as linearity, repeatability and recovery. No interfering peaks showed up in any of the LC-MS/MS trace. CCα and CCβ were similar to those obtained for whole milk powder. CCα and CCβ were 0.024 and 0.031 μg/kg, respectively. Table 3 shows the relative recovery, repeatability and absolute recovery obtained from skim milk samples.

Table 3. Relative recovery, precision and absolute recovery for skim milk powder (n=6)

Spike level (μg/kg)	0.1	0.2	0.3	0.6
Relative recovery (%)	94	105	107	99
RSD (%)	10.7	7.4	6.8	11.2
Absolute recovery (%)	80	78	76	74

CONCLUSIONS

The developed LC-ESI-MS/MS method using Chem Elut extraction cartridge, validated according to European Commission criteria, could be applied to control chloramphenicol in whole milk powder. RSD was lower than 14% and 16% for repeatability and reproducibility. The *Youden* ruggedness test applied to the milk powder preparation procedure showed that the selected potential critical parameters do not significantly affect the assay results. This method can be also applied to skim milk powder. The developed method may be used for the detection of CAP at 0.1 μg/kg.

Conflict of interest

The authors declare no conflict of interest.

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SUGAR AND INORGANIC ANIONS CONTENT IN MINERAL AND SPRING WATER-BASED BEVERAGES

Maciej Bilek,[1] Natalia Matłok,[2] Janina Kaniuczak,[1,3] Józef Gorzelany[2]*

[1] Laboratory for Environment Health Analysis and Materials of Agricultural Origin, Faculty of Biology and Agriculture, University of Rzeszów, Poland
[2] Department of Food and Agriculture Production Engineering, Faculty of Biology and Agriculture, University of Rzeszów, Poland
[3] Department of Soil Science, Environmental Chemistry and Hydrology, Faculty of Biology and Agriculture, University of Rzeszów, Poland

ABSTRACT

Background. Carbonated and non-carbonated beverages manufactured based on mineral and spring waters have been present at the Polish market shortly, and their production and sales are regularly growing. The products have become commonly known as flavoured waters.
Objective. The aim of the work was to identify and assess the content of carbohydrates used for sweetening mineral and spring water-based beverages and to estimate a concentration of inorganic anions.
Material and methods. The study was undertaken for 15 mineral and spring water-based beverages subject to an analysis contents of fructose, glucose and sucrose with the high-performance liquid chromatography method with ELSD detection) and chlorides, nitrates and sulphates contents using the ion chromatography method.
Results. A chromatographic analysis has confirmed the total contents of sugar declared by the manufacturers. The carbohydrates identified included fructose, glucose and sucrose (added sugar). Chlorides and sulphates were found in the content of all the analysed beverages while nitrates were not determined in only one of the 15 examined beverages.
Conclusions. Mass consumption of mineral and spring water-based beverages should be considered as an important source of sugar and their excessive consumption may be disadvantageous for human health. A consumer should be informed by a manufacturer about a daily dose of sugar in a portion of a drink in per cents, and the easiest way to do it is to provide GDA marks on the label. Mineral and spring water-based beverages do not pose threats to consumer health in terms of their contents of inorganic ions: chlorides, nitrates and sulphates.

Key words: *carbonated beverages, carbohydrates, anions, food labeling, nutritional requirements, nutritional value*

STRESZCZENIE

Wprowadzenie. Gazowane i niegazowane napoje, produkowane na bazie wód mineralnych i źródlanych, obecne są na polskim rynku od niedawna, a ich produkcja i sprzedaż systematycznie wzrasta. Produkty te zyskały sobie potoczną nazwę „wód smakowych".
Cel badań. Celem niniejszych badań była identyfikacja i ocena zawartości węglowodanów, użytych do słodzenia napojów na bazie wód mineralnych i źródlanych oraz oszacowanie stężenia anionów nieorganicznych.
Materiał i metody. Materiał do badań stanowiło 5 rodzajów napojów na bazie wód mineralnych i źródlanych, w których zbadano zawartość fruktozy, glukozy i sacharozy metodą wysokosprawnej chromatografii cieczowej (HPLC) z detekcją ELSD oraz zawartość chlorków, azotanów i siarczanów metodą chromatografii jonowej.
Wyniki. Analiza chromatograficzna badanych napojów potwierdziła sumaryczną zawartość węglowodanów deklarowaną przez producentów. Stwierdzono obecność fruktozy, glukozy i sacharozy (cukry dodane). We wszystkich 15 badanych napojach odnotowano zawartość chlorków i siarczanów, natomiast zawartości azotanów nie stwierdzono tylko w 1 napoju spośród 15 badanych.
Wnioski. Masowo konsumowane napoje na bazie wód mineralnych i źródlanych należy uznać za istotne źródło węglowodanów, a zbyt częste ich spożycie może być niekorzystne z punktu widzenia zdrowia człowieka. Konsument powinien być informowany przez producenta o procentowym stopniu realizacji zapotrzebowania na węglowodany w porcji napoju, poprzez umieszczenie na etykiecie oznaczeń GDA. Natomiast napoje na bazie wód mineralnych i źródlanych nie stwarzają zagrożenia zdrowia konsumentów pod względem zawartości anionów nieorganicznych: chlorków, azotanów i siarczanów.

Słowa kluczowe: *napoje słodzone, węglowodany, aniony, znakowanie żywności, wymagania pokarmowe, wartość odżywcza*

***Corresponding author:** Maciej Bilek, Laboratory for Environment Health Analysis and Materials of Agricultural Origin, Faculty of Biology and Agriculture, University of Rzeszów, Ćwiklińskiej Street 2, 35-601Rzeszów, phone, 663-196-847, e-mail: mbilek@univ.rzeszow.pl;

INTRODUCTION

Carbonated and non-carbonated beverages manufactured based on mineral and spring waters have been present at the Polish market shortly, and their production and sales are regularly growing. The products have become commonly known as flavoured beverages. In the context of the EU food legislation, beverages manufactured based on mineral and spring water are not subject to regulations prescribing the quality of those waters [2, 13]. They are solely defined in a food category "Flavoured beverages", and not as "Water, including natural mineral water (...) and spring water and all other bottled or packed waters" [14].

The reputation of popular brands of mineral waters and health resorts producing them is playing a significant role in the growing popularity of such commodities. Such factor is also determining a consumer's approach to this category of beverages, which are regarded as healthy and safe as mineral and spring waters. Their attractive price is also not without significance (as compared to brand soft drinks) and a more favourable taste than the source water. All this is driving the sales of the products higher as opposed to juices, nectars, energy drinks and isotonic sport drinks, and even source mineral and spring waters [3, 7, 10].

A few dozens of such products produced by the majority of mineral and spring water manufacturers are currently available at the Polish market. The manufacturers of such products frequently refer to their health-related properties by highlighting positive characteristics as compared to classical soft drinks [11]. Information can be found on their labels claiming "crystal clear mountain water", "flavour of tasty fruit", "refreshing composition of fruit flavours", etc. Instead, artificial or natural fruit aromas are found in the content of such beverages, sweeteners (Aspartame, Acesulfame Potassium, Sucralose, Sodium Cyclamate, Sodium Saccharin, Steviol Glycosides), antioxidants (ascorbic acid), acidity regulators (citric acid), preservatives (sodium benzoate), and more rarely – fruit juices obtained from concentrated fruit juices.

"Carbohydrates" or "sugar" are often declared on labels as sweeteners, however without specifying, what chemical compounds are involved. It is also note worthy that the labels of mineral and spring water-based beverages do not have the information required for classical mineral and spring waters, in particular what inorganic anions form their part [2, 13].

The aim of this work was to determine what carbohydrates have been used for sweetening mineral and spring water-based beverages and to determine the profile of three inorganic anions (chlorides, nitrates and sulphates) in the investigated samples.

MATERIAL AND METHODS

15 samples of mineral and spring water-based beverages were taken to analysis. The products were bought in shops at the area of Rzeszow.

The Dionex ICS 1000 ion chromatography system, controlled with Chromeleon version 6.8 software, was used for analysing the content of anions in the tested samples. A reference solution, containing seven anions, was sourced from Thermo Scientific company. A mobile phase was prepared by diluting hundred times the initial solution of 0,8M Na_2CO_3/0,1M (from Thermo Scientific), dedicated to the AS 14A analytical column. Isocratic flow with a flow rate of 1 ml per min. was used. Chromatographic separation was carried out by means of the IonPack AS 14A analytical column together with the AS 14G guard column by Thermo Scientific. The column was thermostated at the temperature of 30°C. Conductometric detection was used and the measuring cell temperature was 35°C. The ASRS-4 mm suppressor was used for suppressing the phase conductivity. A data collection rate was set at 5.0 Hz. Data processing was carried out with Chromeleon 6.8 software.

The basic validation parameters of the IC analytical method applied were estimated. The specificity of the method was confirmed through the injection of the set of standards of seven inorganic anions. The linearity of detector response to the set concentrations of standard solutions within the range of 1.0 to 25 $mg \cdot L^{-1}$ was also determined for the three mentioned inorganic anions: chlorides, nitrates and sulphates. The precision of the analytical method described was confirmed by repeating three times the injection of the set of standards and each of the samples. The stability of the chromatographic system was controlled at intervals lasting five hours by injecting the set of standards in which the concentrations of anions corresponded to the results obtained most often for the prepared samples. In addition, each sample was controlled through fortification with the standard of seven anions in order to confirm the identification of the individual anions. The calibration curves were obtained by plotting concentration (in $mg \cdot L^{-1}$) against peak area. Responses obtained in the examined range were expressed by the linear equation $y = ax+b$.

The samples were degassed for 60 minutes prior to a chromatographic analysis. The undiluted samples, before injection onto the chromatographic system, were filtered through the MCE 0.45 μm syringe filters supplied by Alchem.

High-performance liquid chromatography (HPLC) system controlled with Varian Workstation software version 6.9.1, consisting of two high pressure Varian LC 212 pumps, an autosampler Varian ProStar 410, an evaporative light scattering detector Varian ELSD 385

LC and an integrating module Varian Star 800, was used for analysing the content of sugar in mineral and spring water-based beverages. The Cosmosil Sugar-D, 4.6 x 250 mm chromatographic column was used for chromatographic separation. A data collection rate was set at 5.0 Hz. Data processing was carried out with Varian Workstation software, version 6.9.1.

Optimum parameters of the chromatographic analysis were determined. Isocratic flow; mobile phase composition: acetonitrile: water (80:20 v/v); mobile phase flow rate: 1 ml per min.; injection volume: 25 µl; temperature inside the column thermostat: 35ºC; the autosampler tray temperature: 4ºC. The following ELSD detector parameters were used: the flow rate of gas of 1.2 L per min., the nebulizer temperature of 80ºC and the evaporator temperature of 80ºC. Acetonitrile was supplied by the Polish chemical company POCh.

The basic validation parameters of the analytical method applied were estimated. The specificity of the method was confirmed with injections of single standards of the three examined carbohydrates: fructose, glucose and sucrose and their mixture. The linearity of detector response to the set concentrations of standard solutions within the range of 0.5 to 30 $mg \cdot ml^{-1}$ was also determined for the three mentioned carbohydrates. The repeatability of the detector's responses to known concentrations of the studied carbohydrates was controlled by periodic injections of the kit of standards: fructose, glucose and sucrose. The calibration curves were obtained by plotting concentration (in $mg \cdot ml^{-1}$) against peak area. Responses obtained in the examined range were expressed by the linear equation y = ax+b. The standards of carbohydrates were supplied by Sigma Aldrich.

The samples were degassed for an hour prior to a chromatographic analysis and filtered through MCE syringe filters with the pore diameter of 0.45 µm supplied by Alchem.

RESULTS

A note about total carbohydrates and sugar contained was provided on the labels of eleven out of the twelve examined beverages prepared based on mineral water and spring water. The labels informed about the

Table 1. Declared and determined content of carbohydrates in the tested beverages

Name of beverages	Content declared (in 100 ml of beverages)	Content determined			Total sugar [g/100 ml]
		Fructose [$mg \cdot ml^{-1}$] ± SD (n=3)	Glucose [$mg \cdot ml^{-1}$] ± SD (n=3)	Sucrose [$mg \cdot ml^{-1}$] ± SD (n=3)	
Raspberry-flavoured Żywiec Zdrój	Sugar; total carbohydrates of 4.5 g	18.93 ±0.082	19.22 ±0.190	7.24 ±0.052	4.53
Orange-flavoured Żywiec Zdrój	Sugar; carbohydrates 4.9 g incl.sugar 4.9 g	19.47 ±0.255	19.87 ±0.164	12.11 ±0.098	5.14
Apple-flavoured Żywiec Zdrój	Sugar, apple juice of concentrated juice; carbohydrates 4.8 g including sugar 4.8 g	17.67 ±0	17.9 ±0.069	11.88 ±0.081	4.74
Strawberry-flavoured Żywiec Zdrój	Sugar, raspberry juice of concentrated juice; total carbohydrates of 6.0 g incl. sugar 6.0 g	23.61 ±0.015	23.83 ±0.054	11.21 ±0.094	5.86
Lemon-flavoured Żywiec Zdrój	Sugar; total carbohydrates of 5.6 g incl. sugar 5.6 g	21.88 ±0.097	22.15 ±0.171	9.91 ±0.031	5.39
Wysowianka Lemon	Sugar, sodium cyclamate, sodium saccharin; sugar content undeclared	16.47 ±0.043	16.67 ±0.071	7.31 ±0.042	4.04
Strawberry-flavoured Waterrr	Cane sugar, raspberry juice of concentrated raspberry juice, lemon juice of concentrated lemon juice; carbohydrates 5.9 g	19.49 ±0.016	19.29 ±0.056	21.2 ±0.125	5.99
Lemon-flavoured Waterr	Cane sugar, lemon juice of concentrated lemon juice; carbohydrates 4.4 g	6.6 ±0.032	6.6 ±0.056	31.73 ±0.401	4.49
Raspberry-flavoured Arctic Plus	Sugar, aspartame, acesulfame potassium, carbohydrates 2.8 g incl. sugar 2.8 g	5.03 ±0.09	4.98 ±0.067	17.02 ±0.08	2.7
Lemon-flavoured Arctic Plus	Sugar, aspartame, acesulfame potassium, carbohydrates 2.8 g incl. sugar 2.8 g	6.79 ±0.09	6.72 ±0.026	12.97 ±0.125	2.64
Non-carbonated raspberry-flavoured Nałęczowianka	Glucose-fructose syrup and sugar, acesulfame potassium, sucralose; carbohydrates 3.0 g incl. sugar 3.0 g	10.61 ±0.155	12.52 ±0.071	8.04 ±0.038	3.11

Table 2. Declared and determined inorganic anions content in the tested beverages

Name of beverages	Declared anions content	Concentration determined		
		Chlorides [mg·L^{-1}] ± SD (n=3)	Nitrates [mg·L^{-1}] ± SD (n=3)	Sulphates [mg·L^{-1}] ± SD, (n=3)
Non-carbonated lemon-flavoured Nałęczowianka	None	5.00 ± 0.045	1.31 ± 0.011	3.33 ± 0.005
Non-carbonated strawberry-flavoured Aquarel Nestle	None	5.46 ± 0.051	1.37 ± 0.094	4.73 ± 0.043
Non-carbonated strawberry-flavoured Żywiec Zdrój	None	7.47 ± 0.03	4.84 ± 0.059	19.06 ± 0.022
Non-carbonated peach-flavoured Aquarel Nestle	None	5.01 ± 0.012	1.42 ± 0.015	3.53 ± 0.009
Non-carbonated orange-flavoured Żywiec Zdrój	None	4.87 ± 0.019	2.2 ± 0.056	23.09 ± 0.017
Non-carbonated strawberry-flavoured Nałęczowianka	None	5.07 ± 0.024	1.34 ± 0.098	3.26 ± 0.057
Non-carbonated lemon-flavoured Żywiec Zdrój	None	5.38 ± 0.024	5.1 ± 0.009	18.31 ± 0.045
Non-carbonated raspberry-flavoured Żywiec Zdrój	None	4.79 ± 0.019	4.34 ± 0.094	16.11 ± 0.028
Non-carbonated strawberry-flavoured Aqua	None	3.33 ± 0.083	-	16.25 ± 0.084

contents of "sugar", "glucose-fructose syrup" or "cane sugar". The only manufacturer that did not provide contents of sugar on the label was the manufacturer of a carbonated orange-lime-flavoured drink "Wysowianka Lemon" produced based on water from the Wysowa-Zdrój spring; the contents for fructose was, respectively: 1.64 g/100 ml, for glucose: 1.66 g/100 ml, for sucrose: 0.73 g/100 ml. The tests conducted confirmed that the declared amounts of carbohydrates corresponded to the amount determined. The highest disparity recorded (orange-flavoured Żywiec Zdrój drink) did not exceed 250 milligrams for the declared amount of 4.9 g/100 ml and for the determined amount of 5.14 g/100 ml. The presence of three carbohydrates in various quantitative proportions was confirmed in all the tested beverages, i.e.: fructose, glucose and sucrose. The rate of glucose to fructose concentrations was close to 1:1 for all the beverages (Table 1).

The content of chlorides, nitrates and sulphates was determined in the tested beverages. The amount of chlorides varied between 3.33 to 7.47 mg·L^{-1}, nitrates between 1.31 to 5.1 mg·L^{-1} and for sulphates between 3.26 to 23.09 mg·L^{-1} (Table 2).

DISCUSSION

The sugar content in food products, including beverages, has become discussed more and more intensively. Efforts have been taken not only to impose restrictions but also to prohibit the sale of sweetened beverages [7]. The basis of a heated debate is the commonly known impact of sugar on the development of civilisation diseases such as obesity, diabetes, atherosclerosis, hypertension, tumorous diseases, tooth decay [1, 5, 6, 9, 16].

The Nutritional Guidelines for the Polish Population of 2012 provide that the guideline daily amount of sugar should be 130 grams, and the amount of energy coming from the consumed, added sugar should not be higher than 10% of total energy demand [4, 15]. The following three manufacturers: Żywiec Zdrój S.A., Hoop Polska Sp z o.o. and Nestlé Waters Polska S.A. provide on its products GDA (*Guideline Daily Amount*) information indicating a recommended daily amount. A single, quarter-litre portion of a spring or mineral water-based beverages of Żywiec Zdrój S.A. may cover between 4 to 6% of the daily amount for total sugar, between 12 to 17% of the demand for added sugar, i.e. carbohydrates added to food in food production (these include fructose, glucose and sucrose for the product category discussed) and between 2 to 3% of energy demand [15]. Such amounts are, on average, two times lower as compared to classical soft drinks, as, e.g. a quarter-litre portion of a cola drink containing 26.5 g of total added sugar may satisfy as much as 29% of the guideline daily amount of sugar and cover 5% of the energy demand.

The labels of the tested mineral and spring water-based beverages are lacking information about the content of inorganic anions, which is legally required for manufacturers of classical mineral and spring waters [2, 13]. The identified concentrations of inorganic anions did not exceed the permitted standards which, for nitrates, are at the level of 10 mg·L^{-1} (for natural mineral water extracted at the territory of the Republic of Poland) and of 50 mg·L^{-1} (for water intended for human consumption) and at the level of 250 mg·L^{-1} for sulphates and chlorides [12, 13].

CONCLUSIONS

1. Mineral and spring water-based beverages should be considered as an important source of sugar in diet. A high consumption of such products at a rate of one litre per day may easily supply excessive amounts of such nutrients to an organism, hence causing unfavourable effects likely to lead to civilisation diseases.
2. Tested beverages contained fewer added sugar than classical soft drinks (such as cola, tonics, orange beverages do not pose threats to consumer health in terms of their contents of inorganic ions: chlorides, nitrates and sulphates.
3. Care should be taken that consumers can easily differentiate the labels of mineral and spring water-based beverages from classical mineral and spring waters.
4. The best way to attract attention to high contents of sugar in mineral and spring water-based beverages is to provide GDA information on labels indicating the demand for, notably, sugar and energy in per cents when consuming one portion of a given product.

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THE EFFECT OF PHENOBARBITAL ON GENE EXPRESSION LEVELS OF *p53* AND *Dnmt1* IN THE LIVER OF *WISTAR* RATS

Katarzyna Urbanek-Olejnik[*], *Monika Liszewska*, *Grażyna Kostka*

Department of Toxicology and Risk Assessment, National Institute of Public Health-National Institute of Hygiene, Warsaw, Poland

ABSTRACT

Background. Our previous studies have shown that short-term treatment with phenobarbital (PB) resulted in cytosine methylation of CpG sites on the *p53* gene promoter in male *Wistar* rats' liver. Furthermore, PB induced DNA-methyltransferases (DNMTs) activity was also demonstrated; being the enzymes that catalyze methyl group transfer to cytosine in CpG dinucleotides.

Objective. Since DNA methylation is involved in regulating gene transcription and that DNMT1 is implicated in regulating DNA methylation, this study assessed whether PB-induced hypermethylation of the *p53* promoter region was associated with an altered expression of *p53* and *Dnmt1* genes.

Material and methods. Male *Wistar* rats received PB in three daily oral doses (at 24-h intervals) of 92,8 mg/kg b.w. x day^{-1}. Levels of mRNA for *p53* and *Dnmt1* and levels of relevant proteins were respectively examined by Real-Time PCR and Western blot analysis.

Results. Gene expression analysis revealed that exposure of *Wistar* rats to PB caused statistically significant alternations in the expression of tested genes. We found that both mRNA and protein expression of p53 was down-regulated, whereas expression of *Dnmt1* (both mRNA and protein) was up-regulated after PB treatment.

Conclusions. Suppression of *p53* mRNA and protein expression, which is probably a result of epigenetic changes, (in particular aberrant *p53* promoter hypermethylation), can be associated with tumour promoting activity of phenobarbital.

Key words: *phenobarbital, p53, Dnmt1, genes expression, liver, rats*

STRESZCZENIE

Wprowadzenie. Nasze wcześniejsze badania wykazały, że krótkoterminowe narażenie szczurów *Wistar* na fenobarbital (PB) stymulowało metylację cytozyny w badanych sekwencjach rejonu promotorowego genu *p53*. Ponadto stwierdzono wzrost aktywności metylotransferaz DNA (DNMT), enzymów które katalizują przenoszenie grupy metylowej do cytozyny w dinukleotydach CpG.

Cel badań. Z uwagi że metylacja DNA pełni istotną rolę w ekspresji genów, a DNMT1 uczestniczy w regulacji metylacji DNA, w prezentowanych badaniach oceniano czy indukowana PB hipermetylacja rejonu promotorowego genu *p53* była związana ze zmianami ekspresji genów *p53* i *Dnmt1*.

Materiał i metody. Samce szczurów szczepu *Wistar* otrzymywały PB w dawce 92,8 mg/kg m.c. x dzień$^{-1}$, 3-krotnie w odstępach dobowych. Analizę poziomu transkryptów i białek badanych genów przeprowadzano odpowiednio metodą Real-Time PCR i Western blot.

Wyniki. W wyniku oddziaływania PB wykazano obniżoną ekspresję genu *p53* i wzrost ekspresji metylotranserazy 1 (DNMT1).

Wnioski. Supresja ekspresji *p53* (na poziomie mRNA i białka) będąca prawdopodobnie wynikiem zmian epigenetycznych, w szczególności hipermetylacji jego rejonu promotorowego może być związana z promocyjną aktywnością fenobarbitalu.

Słowa kluczowe: *fenobarbital, p53, Dnmt1, ekspresja genów, wątroba, szczury*

*** Corresponding author:** Katarzyna Urbanek-Olejnik, Department of Toxicology and Risk Assessment, National Institute of Public Health-National Institute of Hygiene, Chocimska street 24, 00-791 Warsaw, Poland, phone: +48 22 5421 356, e-mail: kolejnik@pzh.gov.pl

INTRODUCTION

In eukaryotes, it is well known that the degree of chromatin compaction has an inherent role in regulating DNA accessibility for transcription factors. In condensed forms of chromatin (heterochromatin), most genes are inactive. The main mechanisms of regulating this gene expression are DNA methylation and post--transcriptional modifications of histones resulting in the reorganisation of chromatin structure. On the other hand, mounting evidence suggests that epigenetic alterations are early indicators of environmental chemicals' exposure [2, 5, 16].

From the biochemical point of view, DNA methylation is based on the coupling of methyl group to C^5 of cytosine ring found in CpG dinucleotides, where S--adenosyl methionine (SAM) serves as the methyl group donor. The reaction is catalysed by a group of DNA methyltransferases (DNMTs) [6]. In the mammalian cell there are three known active DNMTs (DNMT1, DNMT3a and DNMT3b). Among them, DNMT1 is responsible for maintaining the normal methylation pattern in repeated cycles of replication preceding cell divisions.

In our previous studies [14], during short-term treatment of *Wistar* rats with PB (it being the most widely used anticonvulsant worldwide and classical nongenotoxic rodent liver carcinogen), altered DNA methylation of the *p53* gene was observed. Cytosine hypermethylation in the analysed CpG sites of the *p53* gene promoter was associated with increased DNMTs' activity as well as DNA synthesis.

Since methylation changes in the promoter region of genes is one of the pathways for regulating gene expression [11], the present studies focus on evaluating whether PB-induced hypermethylation of *p53* gene can affect transcriptional activities of this tumour suppressor gene. We thus estimate the effects of PB on *p53* as well as *Dnmt1* expression (both mRNA and protein) in the liver of male *Wistar* rats.

MATERIAL AND METHODS

Animals and treatment

Twenty male *Wistar* rats (aged 5 weeks, 110-130 g) were purchased from the Center of Experimental Medicine, Medical University of Bialystok, Poland. Before treatment, the animals were housed (five rats per cage) at a temperature of 22± 1°C, relative humidity of 50 ±10% and on a 12-h light:12-h dark cycle. The animals were allowed unrestricted access to tap water and a standard rodent diet. The animals were also permitted to acclimatise for at least 2 weeks at the described conditions under which they were maintained throughout the study. Rats weighing 200-220 g were randomly distributed into two groups. PB was administered by oral gavage in an olive oil suspension (92,8 mg/kg b.w. x day^{-1}) between 08:00 h and 09:00 h, for 3 days (at 24-h intervals). Control animals received only the olive oil suspension. Sections of the liver's right lobes were removed, frozen in liquid nitrogen and stored at -80° C.

All procedures involving animals were performed according to national animal welfare regulations after receiving authorisation by the Local Ethic Committee for the conduct of research studies on live vertebrates (No 23/2010).

Real-Time PCR for p53 and Dnmt1 genes

Total RNA was extracted from frozen liver tissue using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. cDNA were synthesised from 1 μg total RNA using Moloney Murine Leukemia Virus reverse transcriptase (Clontech, USA). The cDNA was then analysed by quantitative Real-Time PCR using a KapaSybr®FastqPCR Kit (KapaBiosystems, USA) on the MiniOpticon system (Bio-Rad). The Real-Time PCR conditions and the primer sequences for *Dnmt1* and β-*actin* were described previously [26].

Primers for *p53* were as follows: p53 F-5'TCTGTTTCAAAAAGCAAAAAGATGAC-3', p53 R-5' ATAGCAAGGAAAGTCATGAACTGCCA-3' (GenBank No NM_030989.3).

Western blot analysis of p53 and DNMT1 proteins expression

Western blotting was performed as previously described [26]. The following antibodies were used: p53 (dilution 1:5000), DNMT1 (1:2000), β-actin (1:5000), peroxidase-conjugated goat antimouse IgM antibody and donkey anti-goat IgM antibodies (1:5000 dilution) (Santa CruzBiotechnology, USA). Proteins were resolved by 10% SDS-PAGE and transferred to Immobilon-P membrane (Millipore). Bands were quantified by the Image Quant software (Molecular Dynamics, version 5.2) and normalised relative to β-actin. Fold changes were expressed by normalising the corresponding value of the vehicle-treatment animals to one of the internal controls.

Statistical analysis

Protein levels were analysed by the *Student's t*-test. Statistical analyses were performed using Statistica software (version 6). REST software Randomization tests (Pair Wise Fixed Reallocation Randomization TEST) was used to assess the statistical significance of demonstrated differences in mRNA levels [18]. A probability level less than 0.05 was used as a criterion for significance.

RESULTS

Effect of PB on p53 mRNA and protein expression

Using Real-Time PCR, gene-specific mRNA expression was quantified in the liver from rats treated with PB and results were expressed relative to the number of β-*actin* transcripts. The mRNA level of *p53* from the control group was set at 1.00 and mRNA expression of experimental groups was evaluated by its relative ratio. As shown in Figure 1A, repeated treatment with PB (3 days of dosing), decreased *p53* mRNA in the liver of *Wistar* rats in comparison to their respective vehicle-treated animals. The transcript level of *p53* gene was decreased to approximately 60% of the normalised control level.

Given that PB treatment decreased *p53* gene transcription, we then questioned whether the protein level of this gene was also affected by PB. The densitometric scanning of western blot results showed that protein levels in the liver of rats exposed to PB were also decreased compared to controls. Figure 1B, shows that the protein level was reduced by up to 34% as a result of three PB doses.

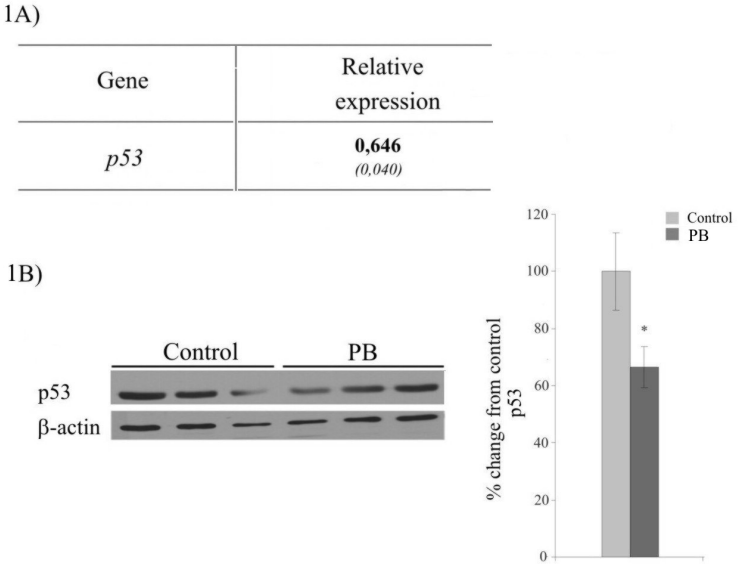
Effect of PB on Dnmt1mRNA and protein expression

A comparison of results from methylation analysis with the results from quantifying the expression of epigenetic modulating enzymes, like DNMTs, can provide a valuable insight into the causes of epigenetic alterations [10].

Since DNMT1 is considered to be primarily responsible for the maintenance of DNA methylation and that it copies the pre-existing methylation pattern onto the daughter strand after DNA replication [13], we have therefore examined DNMT1 expression in PB--treated rats. The effect of PB on hepatic *Dnmt1* mRNA and protein expression is shown in Figure 2.

Figure 2A demonstates that, relative *Dnmt1* mRNA levels were changed significantly as compared to the control animals. The treatment with a tumor-promoting dose of PB for 3 days resulted in up-regulation of *Dnmt1* transcription. A statistically significant difference was evident when comparing the data of the exposed group vs. those of the vehicle control group. We have shown a statistically significant (p=0.04) increase in *Dnmt1* mRNA by over 50%.

An analysis of DNMT1 expression was then performed and increased DNMT1 was detected in response to PB (Figure 2B). DNMT1 protein in rat liver was found to be significantly increased by 22% as compared with controls.



1A) Real-Time PCR analysis was used to determine mRNA levels of p53 gene. The data were normalized to an endogenous reference,β-actin and expressed as relative to control value; p-values are given in brackets; and statistical analyses were carried out using randomization test.

1B) Representative bands obtained by Western immunoblotting analysis of p53. The graphs on the right represent the mean ± S.E.M. from group of five rats (n=5), normalized to β-actin. The mean value of protein, indicated in percentage, relative to controls, considered 100%.

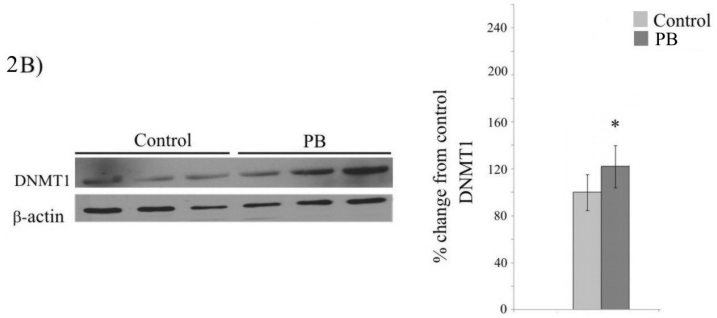
Asterisks (*) indicate statistical difference from the corresponding control group.

Figure 1. The effects of PB on *p53* gene expression (at mRNA and protein level) in the liver of male *Wistar* rats.

2A)

Gene	Relative expression
Dnmt1	**1,575** *(0,040)*

2B)



2A) Real-Time PCR analysis was used to determine mRNA levels of Dnmt1 gene. The data were normalized to an endogenous reference, β-actin and expressed as relative to control value; p-values are given in brackets; and statistical analyses were carried out using randomization test.

2B) Representative bands obtained by Western immunoblotting analysis of DNMT1. The graphs on the right represent the mean ± S.E.M. from group of five rats (n=5), normalized to β-actin. The mean value of protein, indicated in percentage, relative to controls, considered 100%.
Asterisks (*) indicate statistical difference from the corresponding control group.

Figure 2. The effects of PB on DNA methyltranferases 1 (DNMT1) expression in the liver of male *Wistar* rats.

DISCUSSION

The *p53* is one of the most frequently altered tumor suppressor genes in cancer [3]. It is also well known that mutations in *p53* result in loss-of-function. In addition to this genetic inactivation, epigenetic mechanisms also contribute to the inactivation or down-regulation of tumor suppressor genes, including *p53*[21]. The encoded p53 protein, which is ubiquitously expressed in tissue, keeps genome stability under stress, and is involved in multiple cellular activities. This protein plays a key role in the regulation of the cell cycle, DNA repair and apoptosis [22, 23]; these biological processes being critical for the initiation of carcinogenesis [4].

DNA methylation is one of several epigenetic mechanisms that cells use to control gene expression and DNA hypermethylation acts as an alternative mechanism for inactivating tumor suppressor genes [10].

Previously, we had examined methylation status of *p53* in cytosine residues located at nt: -450, -261, and -179 and that phenobarbital (PB) was found to stimulate *p53* promoter hypermethylation [14]. Because it is known that DNA methylation and gene expression are closely linked [11], in the way that methylation can lead to inappropriate gene silencing [9, 24], a Real-Time PCR and western blot was undertaken to respectively measure levels of the *p53* transcript and protein. Our results indicate that expression of *p53* was down-regulated after short-term exposure (3 days) of the animals to PB. A concomitant decrease in expression of p53 protein was also observed, confirming that the decreased RNA expression translated into a decrease in protein expression. This suggests that an epigenetic component such as promoter methylation might play an important role in regulating *p53* expression.

Some studies have documented a correlation between aberrant *p53* gene promoter methylation with low levels of mRNA production. This relationship was reported in human primary hepatocellular carcinoma [20] and in lymphoblastic leukemia [1]. In contrast, some studies [15, 25] suggest that other epigenetic modifications might be involved in regulating the *p53* gene. On the other hand, there is evidence on the importance of hypermethylation in the non-CpG island-containing promoter coding region in gene inactivation [19]. Within this context, it should be noted that the *p53* promoter region does not contain a CpG island and therefore, may be more sensitive to site-specific methylation [12, 20].

We have also demonstrated a PB-mediated significant increase in *Dnmt1* mRNA and protein levels. Increased expression of DNMT1 has been reported in development of hepatocellular [8] and pancreatic carcinomas [17]. Furthermore, it has been reported that DNMT1 induces hypermethylation of tumor suppressor genes to epigenetically mediate their repression [7]. This leads to the assumption that PB affects hypermethylation of *p53* through its positive regulation of DNMT1.

In conclusion, suppression of *p53* mRNA and protein expression, (which is probably a result of epigenetic

changes and, particulary aberrant *p53* promoter hypermethylation), can be associated with tumor promoting activity of phenobarbital.

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Conflict of interest
The authors declare no conflict of interest.

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ADAPTATION OF A NEUROBEHAVIORAL TEST BATTERY FOR THAI CHILDREN

Juthasiri Rohitrattana[1], Wattasit Siriwong[1, 3], Panrapee Suittiwan[2], Mark G. Robson[3, 4, 5], Pam Ohman Strickland[4], Diane S. Rohlman [6, 7], Nancy Fiedler[4]*

[1] College of Public Health Sciences Chulalongkorn University, Institute Building 2-3, Soi Chulalongkorn 62, Phyathai Rd, Pathumwan, Bangkok 10330, Thailand
[2]Faculty of Psychology Chulalongkorn University, Wittayakitt Building Fl.16, Siam Square soi 64 Payathai Rd., Patumwan, Bangkok 10330, Thailand 10330
[3] Thai Fogarty ITREOH Center, Chulalongkorn University, Bangkok, Thailand
[4] Environmental and Occupational Health Sciences Institute, Rutgers University, 170 Frelinghuysen Rd., Piscataway, NJ 08854, USA
[5] School of Environmental and Biological Sciences, Rutgers University, 59 Dudley Road New Brunswick, NJ 08901, USA
[6] Department of Occupational and Environmental Health - CPHB, Suite 300, 145 N. Riverside Dr., The University of Iowa, Iowa City, IA 52242, USA
[7] Center for Research on Occupational and Environmental Health, Oregon Health and Science University, 3181 SW Sam Jackson Park Road - L606, Portland, OR 97239, USA

ABSTRACT

Background. Exposure to neurotoxicants is a world wide problem with significant health implications for child development. In spite of higher neurotoxicant exposures, many developing countries do not have established neuropsychological instruments.

Objective. This study evalauted the adaptation and reliability of a computer and examiner administered Behavioral Assessment and Research System (BARS) that includes tests of motor speed and dexterity, attention, memory, and visuospatial coordination for use in Thailand.

Material and methods. To assess test-retest and alternate form reliability, BARS was administered to 24 healthy, 6-8 year old urban Thai children during two testing sessions two weeks apart. A comparison group of 29 healthy, rural Thai children of similar age and sex completed the BARS as part of another study and comprised a comparison group.

Results. Test-retest reliabilities for tests without alternate forms ranged from 0.41 to 0.77, but reliabilities were lower for tests with alternate forms (0.11 to 0.83). Paired t-tests revealed few significant differences in group performance between test administrations. Performance of urban Thai participants was compared to 29 rural Thai participants of similar age and sex. Parental education was significantly greater for urban vs. rural participants, resulting in signficant differences in performance on tests of motor speed.

Conclusions. This study supports the use of BARS for epidemiologic studies of neurotoxicants in Thailand, but highlights the sensitivity of these tests to differences in parental education and the need for improved alternate test forms.

Key words*: neurobehavioral tests, children, Thailand, reliability, Behavioral Assessment and Research System*

STRESZCZENIE

Wprowadzenie. Narażenie na substancje neurotoksyczne jest problemem ogólnoświatowym mającym istotne konsekwencje zdrowotne dla rozwoju dzieci. Wiele państw rozwijających się nie przygotowało narzędzi do badań neuropsychologicznych, mimo występowania dużego narażenia na substancje o działaniu neurotoksycznym.

Cel badań. W badaniach przeprowadzonych przez ankieterów z zastosowaniem komputerów oceniono przystosowanie i wiarygodność testu *Behavioral Assessment and Research System* (BARS), który obejmuje badanie szybkości i zręczności motorycznej, uwagi, pamięci i koordynacji wzrokowej w celu zastosowania w Tajlandii.

Materiał i metody. W celu oceny wiarygodności testu BARS metodą test-retest i zapisu alternatywnego poddano badaniu 24 zdrowych dzieci tajlandzkich w wieku 6-8 lat zamieszkujących w mieście. Ponowne badania przeprowadzono w odstępie 2 tygodni. Grupę porównawczą stanowiło 29 zdrowych dzieci mieszkających na wsi.

***Corresponding author**: Nancy Fiedler, Environmental and Occupational Health Sciences Institute, Rutgers University, 170 Frelinghuysen Rd., Piscataway, NJ 08854, USA; phone: (848) 445-6081, e-mail: nfiedler@eohsi.rutgers.edu

Wyniki. Wiarygodność poszczególnych skal badana metodą *test-retest* rozciągała się od 0,41 do 0,77. Wiarygodność była niższa w przypadku metody zapisu alternatywnego (0,11 do 0,83). *Test t* dla zmiennych połączonych wykazał nieliczne różnice pomiędzy wynikami grupy w dwóch badaniach. Porównanie wyników uzyskanych przez dzieci tajlandzkie mieszkające w miastach i na wsi wykazało wpływ wykształcenia rodziców, które było znacząco wyższe u tych pierwszych.
Wnioski. Badania potwierdzają użyteczność testu BARS do badań epidemiologicznych substancji neurotoksycznych w Tajlandii, ale uwydatniły czułość testu na różnice w wykształceniu rodziców i potrzebą polepszenia zapisu alternatywnego testu.

Słowa kluczowe: *testy neurobehawioralne, dzieci, Tajlandia, wiarygodność, Behavioral Assessment and Research System*

INTRODUCTION

Environmental health issues are a world health concern not only in industrialized countries but also areas of developing countries such as Thailand [8]. Adverse effects on cognitive development among children from industrialized countries have been demonstrated for exposures to a number of neurotoxicants such as lead, mercury, and pesticides [4, 6, 10, 13, 16]. Arguably, children from developing countries are being exposed to higher concentrations of neurotoxicants than in the developed world [14], but few studies have been conducted to evaluate the impact of these exposures on children's cognitive function. The dearth of neurobehavioral research in developing countries is due, in part, to a lack of culturally relevant test batteries. The purpose of the current study is to evaluate the adaptation of a neurobehavioral battery for use in Thailand.

Few tests are available to evaluate cognitive and psychomotor function of Thai children. The *Wechsler* Intelligence Scale for Children (WISC) is a widely used intelligence test that has been adapted primarily for clinical assessment of Thai children [3]. The WISC is lengthy, must be administered by a psychologist, and is not suitable for field epidemiologic studies of children. A small number of studies have used the human figure drawing test, the Test of Nonverbal Intelligence version 3 or standard tests of math and verbal fluency to estimate intelligence among Thai children [9, 15, 21]. Relatively more neuropsychological tests, however, are adapted to screen for dementia among the elderly in Thailand rather than for assessment of children [11].

The Behavioral Assessment and Research System (BARS), originally developed for neurobehavioral evaluation of adults, has been adapted for use in children from 5 years old [17]. This test battery is economical, requires limited language and education abilities, and has been translated into multiple languages to include Spanish, Portuguese, Arabic, and Korean. The BARS has been used for many studies in adults, adolescents, and children in the U.S. and in developing countries and has demonstrated utility for making cross-cultural comparisons of performance [1, 5, 12, 18, 20]. *Farahat* et al [7] reported test-retest reliabilities for BARS administered to adults residing in the United States ranging from 0.35 to 0.85 while *Rohlman* et al [20] reported similar one-month, test-retest correlations for 4 to 9 year old Hispanic non-English speaking children. Neurotoxicant exposures may affect cognitive and motor skills differentially depending on the developmental stage at which exposure occurred, the frequency of exposure and a host of other variables that make it difficult to separate acute, temporary effects from persistent decrements in function. Therefore, repeated assessment of children's cognitive and motor skills is often desirable, particularly in situations where intermittent acute exposures occur in the context of chronic background exposure such as those seen with pesticides in farming communities. Using the same tests allows direct comparisons over time and in differing exposure scenarios, but practice effects may hinder the sensitivity of the tests for detecting subtle behavior change. Therefore, the purpose of the current study is to 1) demonstrate the utility and test-retest reliability of BARS for Thai children, 2) to develop and assess the performance of alternate forms of those BARS tests vulnerable to practice effects, and 3) to compare performance of urban and rural Thai children.

MATERIAL AND METHODS

Participants

To assess the suitability, test-retest reliability, and alternate form reliability of the testing battery. Twenty--four healthy 5 years, 10 months to 8 years, 11 months Thai children from Bangkok (urban sample) volunteered to complete the test battery. The study was explained fully to parents who signed the consent form and the participating children gave verbal assent prior to participation. The study was reviewed and approved by the Institutional Review Boards of Chulalongkorn University and Rutgers-Robert Wood Johnson Medical School.

Neurobehavioral Tests

The following tests (Table 1) were presented on a computer screen equipped with a 9 BUTTON response unit: finger tapping (TAP), match-to-sample (MTS), symbol digit (SD), and the continuous performance test (CPT). In addition digit span (DST) and Object Memory (OMT) were administered by an examiner

Table 1. Description of neurobehavioral tests and functions for BARS

Test Description	Function	Variables
Finger tapping (TAP) • Right and left hand taps for 20 seconds; 2 trials/hand	Response speed and coordination	• Average number of taps each hand
Divided attention (DAT) • Tap while reciting nursery rhyme (Chang song)	Divided attention	• Average number of taps each hand while singing
Purdue pegboard (PEG) • Number of small pegs placed in holes during two 30 second trials each hand • Preferred, non-preferred, and both hand trials	Dexterity	• Average number of pegs placed: preferred, non-preferred, both
Visual motor integration (VMI) • Copied line drawing	Hand-Eye coordination	• Total score for correct segments
Digit span (DST) • Spoken presentation of number sequences ○ Forward and reverse recall	Memory and attention	• raw score maximum digits forward, backward
Object memory test (OMT) • Show and name 16 objects • Immediate and delayed recall Recognition of target and non-target items	Recall and recognition memory	• Immediate recall; delayed recall; recognition
Symbol-Digit (SDT) ○ Match number and symbol from key	Information processing speed	• Average latency (ms) of response for correct match
Match-to-Sample (MTS) • 15 stimuli shown for 3 seconds • Identify target from 3 choices ○ Delay between presentation and choice varies from 1 to 8 seconds	Visual memory	• Average latency (ms) for correct choice • Number correct
Continuous performance (CPT) • Different shapes shown rapidly for 4 min in original version and 7 min in alternate version • Press key when target (original = circle; alternate = triangle) shown	Sustained attention	• Percent correct • Average latency (ms) for correct response (hit) • Average latency (ms) for false alarms • D-Prime

Adapted in part from *Rohlman* et al [20].

as were the following tests adapted from the Pediatric Environmental Neurobehavioral Test Battery (PENTB) [2]: Purdue pegboard (PEG), visual motor integration (VMI), and divided attention (DAT).

Cultural adaptation

Some parameters in the neurobehavioral tests were adjusted or substituted for items familiar to Thai children. For example in the OMT which uses common objects, paper was substituted for envelope because envelopes are not familiar to young Thai children. In addition, hair brush was changed to a hair clip because hair brush has the same pronounciation in Thai as toothbrush; fork was also substituted for the chopstick and ruler for pen. The "Happy Birthday song" used in the DAT was changed to "Chang song", a song about elephants that is common in Thai kindergarten. Video and audio instructions for all tests were translated into Thai.

To reduce practice effects, alternate forms were developed for the following: OMT, SD, MTS, and CPT. A second set of objects regarded as familiar to Thai children were selected for the OMT. The stimulus in CPT was a circle in the original test and a triangle in the alternate test. For CPT, the original version used 100 trials while the alternate was 200 trials to determine the effect of prolonged test time on reliability. Target samples were changed for all trials of the alternate version of MTS. For SD, the pairing of each symbol and number were changed for the alternate version. It was not possible to develop an alternate form of the motor/visuomotor tests, i.e. PEG, TAP, and VMI. There are no alternate items for VMI and therefore, the same items were administered during both test sessions.

The test administrators were doctoral and masters students from the College of Public Health Science and Faculty of Psychology, Chulalongkorn University. All examiners were trained during three separate sessions at least 3 times prior to test administration with the children. The training scheme included a brief introduction to neurobehavioral tests, purpose of each test, and demonstration of proper test administration. During the second training session, the tester practiced the test with their colleagues. They were taught how to troubleshoot the test administration, and what they should say in response to subject performance and questions during the test. For example, they were instructed not to indicate if an answer was "correct" or "wrong", but instead to use phrases such as "go on", "keep trying", "try more" to encourage the child's persistence with the test.

Table 2. Mean and standard deviation, paired t-test, and correlations of test-retest scores with no alternate BARS form (n = 24)

Test	Variables	Test (T_1)		Test (T_2)		Paired t-test	Correlation	
		Mean	S.D.	Mean	S.D.	p-value	*r*	p-value
TAP	Right hand	75.40	7.30	76.90	6.30	0.166	0.71	<0.001
	Left hand	66.70	9.40	66.60	8.90	0.965	0.72	<0.001
DAT: song	Tap right average	56.70	7.30	56.90	5.70	0.853	0.67	<.001
	Tap left average	51.00	8.30	50.70	6.80	0.746	0.77	<0.001
PEG	Preferred hand	13.60	2.10	13.80	1.80	1.000	0.72	<0.001
	Non-preferred hand	12.70	2.10	12.50	1.60	0.890	0.71	<0.001
	Both hands	10.50	1.70	10.50	1.50	0.870	0.71	<0.001
VMI	Total Correct	16.00	1.60	16.60	1.30	0.022	0.64	<0.001
DST	Maximum digits forward	7.09	1.59	7.04	1.27	0.900	0.41	0.047
	Maximum digits backward	3.08	0.97	2.96	0.75	0.500	0.48	0.018

Procedure

Participants were tested twice, 2 weeks apart. Order of the original and alternate test form was counterbalanced and a different tester administered the two versions of the test for each child. All neurobehavioral tests were completed in a quiet classroom with one child tested at a time. In each test station, an examiner gave the child instructions. A trained examiner observed the child perform the practice tests to be certain the child understood the instructions and clarified instructions if the child did not perform the test correctly. The examiner also provided encouragement to maintain the child's attention to the test. Each child had a 10 minute break between completion of the computerized tests and non-computerized tests. After the participant accomplished all neurobehavioral tests, the child received a small gift as a reward.

Statistical Analysis

The statistical analyses were performed using SAS for Windows, version 9.3. Mean and standard errors were calculated for each variable assessed. Pearson product-moment correlations and paired t-tests were used to assess test-retest and alternate form reliability and the effects of age and sex. The Folded F- statistic was used to assess equality of variances followed by ANCOVA with adjustment for age to compare urban and rural Thai subjects' performance.

RESULTS

The average age of the Thai participants was 7.4 years (SD = ±0.85) with an equal number of participants at each age, grade level and sex. All children were native Thai speakers.

Test-retest results

All Thai children completed all tests. Means, standard deviations, p-values of mean differences and correlation coefficients are presented in Table 2. For tests without alternate forms, paired t-tests revealed no significant differences between tests administered at time 1 and 2 with the exception of VMI. VMI performance significantly improved from the first to the second testing session. All test-retest reliabilities were significant for tests without alternate forms ranging from $r = 0.41$ to 0.77.

Alternate form test results

For tests with alternate forms, paired t-tests revealed no significant differences between the alternate forms

Table 3. Mean and standard deviation of original and alternate BARS scores (n=24).

Test	Variables	Original form		Alternate form		Paired t-test	Correlation	
		Mean	SD	Mean	SD	p-value	*r*	p-value
OMT[a]	Immediate recall	8.29	1.81	7.63	2.16	0.175	0.32	0.129
	Delay recall	6.71	2.03	6.83	2.20	0.825	0.16	0.445
	Recognition	15.75	0.44	15.63	1.06	0.543	0.35	0.094
SDT	Latency (ms)	3947.20	1287.10	4107.60	1048.70	0.954	0.83	<0.001
MTS	Latency (ms)	3673.00	613.30	3601.20	519.60	0.486	0.63	0.001
	Correct	12.30	1.70	12.70	1.30	0.278	0.11	0.603
CPT	Percent Hits	0.84	0.19	0.84	0.13	0.897	0.78	<0.001
	HitLatency (ms) (1st 100 trials of alternate)	453.40	86.00	513.00 (488.70)	122.10 (120.80)	0.004 (p=0.10)	0.65	<0.001
	Percent False Alarms	0.11	0.09	0.09	0.08	0.142	0.77	<0.001
	FALatency (ms)	410.70	163.20	505.00	200.20	0.041	0.33	0.118
	Correct Dprime	2.70	1.20	2.70	0.90	0.990	0.81	<0.001

[a] All items included

with the exception of CPT hit latency and CPT latency for false alarms (i.e., incorrect responses). Speed of response was slower for the CPT alternate version in which the number of trials completed by subjects was increased (i.e., 200 trials) (Table 3). A paired t-test comparing performance on the first 100 vs. second 100 trials of the CPT alternate form revealed that hit latency was significantly slower during the second 100 trials (1st 100 mean = 489.0 (SD = 121); 2nd 100 mean = 540.2 (SD = 139); p = 0.005), but false alarm latency was not significantly different (p = 0.55). When hit latency for the original form (i.e., 100 trials) was compared to the latency for the first 100 trials of the alternate form, hit latency was not significantly different. Original and alternate forms of SDT, MTS, and CPT were significantly correlated for latency of correct responses and for percent hits on CPT (r = 0.63 to 0.81). Although speed of response for MTS was highly correlated between

Table 4. Age-adjusted covariance analyses of BARS performance: Thai urban (n=24) vs. rural (n=29)

Variable	Group	Mean	Stanadard deviation	F value	p value
MTS_Correct	urban	12.25	1.67	2.23	0.141
	rural	10.83	2.94		
MTS_Latency (ms)	urban	3676.90	625.00	0.09	0.769
	rural	3857.70	650.00		
TAP_R	urban	76.10	7.54	3.61	0.063
	rural	68.35	12.20		
TAP_L	urban	66.90	9.39	5.90	0.019
	rural	57.48	11.47		
CPT_percent Hits	urban	0.84	0.19	0.22	0.638
	rural	0.80	0.18		
CPT_percent FA	urban	0.11	0.09	0.38	0.540
	rural	0.13	0.10		
CPT_Hit Latency (ms)	urban	453.40	86.03	0.90	0.348
	rural	498.70	139.20		
CPT_FA Latency (ms)	urban	410.70	163.20	0.21	0.646
	rural	464.30	223.20		
Correct_Dprime	urban	2.66	1.17	0.96	0.332
	rural	2.28	0.87		
DAT_R	urban	56.71	7.32	5.30	0.026
	rural	48.21	12.04		
DAT_L	urban	51.02	8.32	4.03	0.050
	rural	55.14	9.59		
DST Maximum Digits Forward	urban	7.08	1.59	2.89	0.095
	rural	6.24	1.43		
OMT_Immediate	urban	8.29	1.81	2.99	0.090
	rural	6.48	3.19		
OMT_Delay Recall	urban	6.71	2.03	2.27	0.138
	rural	5.31	2.75		
OMT_Recognition	urban	15.75	0.44	3.09	0.085
	rural	13.69	4.20		
PEG_Pref	urban	13.58	2.09	2.55	0.117
	rural	12.14	1.90		
PEG_nonPref	urban	12.71	2.09	7.68	0.008
	rural	10.88	1.47		
PEG_B	urban	21.42	3.49	3.50	0.067
	rural	18.62	3.29		
VMI	urban	16.00	1.56	0.11	0.738
	rural	15.69	2.14		

Analyses adjusted for age

the original and alternate stimuli, the number of correct responses was not, suggesting that even though the alternate forms did not affect the overall group mean, individuals did not perform similarly on the original and alternate forms of MTS. Thus, further work will be required to insure the equivalence of the items.

For OMT, scores were not significantly different between the alternate forms, but correlations were low and non-sigificant, suggesting that individual subjects did not perform similarly in response to the alternate forms. Item analyses were conducted by assessing the number of children who correctly remembered each item at both immediate and delayed recall for each form of the test. The number who correctly recalled each item immediately and after a delay was used to predict the total test score for the test version in which that item was administered and the total test score for the alternate test or the test in which the item was not given. Using regression models, items that poorly predicted the alternate form total score for immediate and delayed memory were removed (i.e., negative beta weight) (i.e., comb, ball, and paper from original test; ice cream, plane, and bottle from alternate version). The correlation between immediate memory ($r = 0.41$; $p = 0.05$) and delayed memory ($r = 0.44$; $p = 0.03$) scores for these modified versions were both significant. Paired t-tests comparing mean performance between these versions were not significantly different for immediate (immediate: original mean = 6.88 (SD = 1.60); alternate mean = 6.58 (SD = 2.06)) or delayed memory (delayed: original mean = 5.17 (SD = 1.8); alternate mean = 5.96 (SD = 2.10)).

Age and sex effects

Only tests of motor speed and dexterity, and visual motor integration were significantly correlated with age (PEG right hand: $r = 0.65$; left hand: $r = 0.73$; both hands: $r = 0.73$; VMI: $r = 0.47$) while the remainder of the tests were not significantly affected by age. Females had significantly more correct responses on MTS (female mean = 12.58 (SD = 1.74); male mean = 12.38 (SD = 1.28); $p = 0.03$). They also were significantly faster in coding digits with symbols (SDT) (female mean = 3617.30 (SD = 768.77); male mean = 4280.66 (SD = 1043.13)).

Comparison to rural Thai children

Twenty-nine, healthy 6 to 8 years, 5 month old children from a rural shrimp farming community (rural sample) also completed the original version of the BARS battery. These subjects were recruited as a control group for a study evaluating the cognitive and motor effects of pesticide exposure. The same procedures were used to evaluate this group of children during a single testing session. Because they were of similar age, education level, and sex, comparison with the performance of urban Thai children provides another evaluation of the utility of this testing protocol for use in Thailand.

The urban and rural subjects did not differ in sex distribution, but the urban sample was significantly older (urban mean: 89.46 (SD = 11.77) months; rural mean: 82.72 (SD = 9.52) months; $p < 0.03$) and their parents were significantly more educated (urban mean: 14.29 (SD = 2.80) years; rural mean: 8.97 (SD = 4.53) years; $p < 0.001$) than the rural sample. Therefore, an analysis of covariance adjusting for age was used to compare performance of these groups on the original BARS battery of tests (Table 4). Urban subjects exhibited significantly greater motor speed for the right and left hand (TAP, DAT, PEG) than rural subjects, but no other significant differences were observed. Because the distibution of parental education was remarkably different between the urban and rural sample, a subset of rural children whose parents had > 9 years of education were selected (n = 14) to compare performance with the urban sample (n = 24). After adjusting for age, no significant differences in test performance were observed between the urban and a subset of the rural subjects with comparable parental education (data not shown).

DISCUSSION

Thai children were able to complete all neurobehavioral tests with few significant differences in performance over a two week period, suggesting that BARS has utility for epidemiologic studies where repeated testing of Thai children is needed. As a group the children's mean performance was comparable between the first and second testing sessions on all but visual motor integration and latency of response for continuous performance. Hand-eye coordination (VMI) showed significant improvement on re-testing probably because of familiarity with the figures to be copied while the increased number of trials for the alternate form of CPT contributed to differences in performance. When the 1st 100 trials for the alternate version of CPT was compared to the 2nd 100 trials, significant slowing of response (i.e., latency of hits and false alarms) was observed, suggesting that fatigue may have been a factor. Moreover, when the first 100 trials of the alternate version were compared to the original CPT with only 100 trials, differences in latency were no longer significant. With the exception of object memory (OMT) and number correct in match to sample (MTS), test-retest reliabilities were similar to those cited previously among 4 to 9 year old Hispanic children from the U.S. [20]. Both OMT and MTS used alternate stimuli which likely contributed to lower correlations between test administrations and therefore, further work will be required to insure comparability of these alternate forms.

Repeated testing did not result in signficant changes in group performance for tests of motor speed to include tapping with and without distraction (TAP; DAT) and fine motor manipulation (PEG). However, future studies where repeated testing is planned should consider developing alternate figures for VMI to reduce practice effects. Accuracy in sustained attention (percent hits for CPT), speed and accuracy of information processing (latency for correct SDT responses), and accuracy of immediate memory (maximum digits for DST) were comparable between testing sessions with children exhibiting consistent performance ($r = 0.41 - 0.83$). Thus although children performed more slowly when given more trials for CPT, their accuracy remained consistent.

Group performance for immediate and delayed recall and recognition of objects (OMT) was also similar for alternate forms of the test, but the relative performance of each child as revealed by low test-retest reliabilities was inconsistent. The regression analysis revealed improved test-retest correlation for both immediate and delayed memory when items that were poor predictors of each version were excluded from the total. Testing with a different sample of children will be required to cross-validate this result, but further work on alternate form development would enhance the applicability of this test for sitatuions when repeated testing is required.

Within this relatively restricted age range, only motor (PEG) and visual motor tests (VMI) showed improvement with age. Females exhibited faster information processing (SDT) and more accurate visuospatial processing (MTS) than males. These findings are consistent with *Rohlman's* observation that Filipino, Spanish and English speaking females performed better than males of the same age although they did not observe significant differences [19].

After adjustment for age and parental education, urban and rural Thai children performed similarly on BARS. Even when parental education was not controlled, performance of the urban and rural samples was quite similar except for speed of motor performance suggesting that although parental education was much greater in the urban sample, this made little difference in the performance of the children. Moreover, rural Thai children have much less access to extracurricular activities and their socioeconomic status is generally lower than urban children. Nevertheless, their performance was comparable further validating the suitability of BARS for children of very different cultural and socioeconomic background.

At this stage of development BARS is suitable for epidemiologic studies of children who may be exposed to neurotoxicants or for other group comparisons, but cannot be used to predict or evaluate an individual child's performance. This study lends support to the use of BARS across diverse cultures, socioeconomic groups, and languages which can help strengthen our ability to aggregate data from disparate samples toward understanding neurodevelopment in a variety of circumstances.

CONCLUSIONS

This study demonstrates the utility of using BARS to assess cognitive and motor performance in Thai children. Overall test-retest reliability is acceptable although alternate forms need further refinement to improve their comparability. As for most neurobehavioral tests, age, sex, and parental education influence performance and must be controlled for in the design and analysis of these tests. Continuing cross cultural validation of the BARS will allow data aggregation for the purpose of assessing the effects of world wide neurotoxicant exposure on child development.

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Conflict of interests

OHSU and Dr. Rohlman have a significant financial interest in Northwest Education Training and Assessment, LLC, a company that may have a commercial interest in the results of this research and technology. This potential conflict of interest was reviewed and a management plan approved by the OHSU Conflict of Interest in Research Committee was implemented.

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INFLUENCE OF DAILY DIET ON ASCORBIC ACID SUPPLY TO STUDENTS

Małgorzata Szczuko[1], Teresa Seidler[3], Ewa Stachowska[1], Krzysztof Safranow[2], Maria Olszewska[2], Katarzyna Jakubowska[2,4], Izabela Gutowska[1], Dariusz Chlubek[2]*

[1]Department of Biochemistry and Human Nutrition, Pomeranian Medical University in Szczecin, Poland
[2]Department of Biochemistry and Medical Chemistry, Pomeranian Medical University in Szczecin, Poland
[3]Department of Human Nutrition, West Pomeranian University of Technology in Szczecin, Poland
[4]College of Health, Beauty Care and Education in Poznan, Faculty in Szczecin, Poland

ABSTRACT

Background. Researchers suspect that the accepted adequate ascorbic acid plasma concentration is not being met even after dietary intake of the recommended amount of vitamin C. Current dietary intake recommendation in Poland is 60 mg per day for women and 75 mg per day for man (EAR), while in Western Europe and North America is higher and amounts to 75-90 mg per day.

Objective. The paper aimed at studying a correlation between composition of nutrients in daily diet and plasma vitamin C levels in university students.

Materials and methods. This study examined diet composition and the nutritional status of ascorbic acid in plasma of 120 university students in Szczecin, Poland. Ascorbic acid was determined in blood plasma using HPLC method. The information concerning diet composition was collected using the method of "7-days food record" prior to blood collection.

Results. Plasma ascorbic acid deficiency (<40 μmol/L) was observed in 23% of women and 28% of men. The average plasma ascorbic acid concentration was 48.65 μmol/L in women and 45.61 μmol/L in men. The average intake of vitamin C in women with observed deficiency was average 46.55 mg/day, whereas in men it was 48.56 mg/day.

Conclusions. The recommendation of dietary intake of vitamin C in Poland is low in comparison to other countries. Population-based studies are necessary to determine the actual demand for vitamin C in various population groups in Poland.

Key words: *nutrition, vitamins, dietary intake, diet, ascorbic acid, plasma,*

STRESZCZENIE

Wprowadzenie. Badacze podejrzewają, że przyjęte odpowiednie stężenie kwasu askorbinowego w osoczu nie jest spełnione nawet po spożyciu zalecanej ilości witaminy C. Aktualna norma spożycia witaminy C w Polsce wynosi 60 mg/dzień dla kobiet i 75 mg/dzień dla mężczyzn (EAR), podczas gdy w Europie Zachodniej i Ameryce Północnej jest większa i wynosi 75-90 mg dziennie.

Cel badań. Celem pracy było zbadanie korelacji między składnikami pokarmowymi całodziennej racji pokarmowej a witaminą C u studentów.

Materiał i metody. Badano skład diety oraz zawartość witaminy C w osoczu 120 studentów ze Szczecina. Stężenie kwasu askorbinowego oznaczano w surowicy krwi za pomocą HPLC. Informacje dotyczące żywienia zebrano stosując metodę 7 dniowego zapisu żywieniowego przed pobraniem krwi.

Wyniki. Niedobór kwasu askorbinowego w osoczu (< 40 μmol/L) wystąpił u 23% kobiet i 28% mężczyzn. Średnie stężenie kwasu askorbinowego w osoczu wynosiło 48.65 μmol/L u kobiet i 45,61 μmol/L u mężczyzn. U kobiet, u których stwierdzono niedobór witaminy C, jej średnie spożycie z dietą wynosiło 46,55 mg/dzień, podczas gdy u mężczyzn 48,56 mg/dzień.

Wnioski. Zalecana w Polsce norma dziennego spożycia witaminy C jest niska w porównaniu z innymi krajami. Konieczne są badania populacyjne w celu określenia rzeczywistego zapotrzebowania na witaminę C wśród różnych grup społecznych w Polsce.

Słowa kluczowe*: żywienie, witaminy, spożycie, dieta, kwas askorbinowy, osocze*

***Corresponding author**: Małgorzata Szczuko, Department of Biochemistry and Human Nutrition Pomeranian Medical University, Broniewskiego Str. 24, 77-460 Szczecin, Poland, phone: +48 91 441 48 06,
e-mail: malgorzata.szczuko@pum.edu.pl

INTRODUCTION

The discovery of ascorbic acid was associated with the occurrence of scurvy, observed as early as the middle ages, mainly in the northern regions of Europe. These areas of cool temperate zone, were deficient in fresh fruit and vegetables in winter and hence residents had a diet poor in ascorbic acid. This avitaminosis could be found among people and animals which in the course of evolution lost their ability to synthetize L-ascorbic acid in their body, due to the absence of L-gulono-γ-lactone oxidase in their liver [5].

Ascorbic acid is absorbed from the intestine in higher concentrations by passive diffusion and in lower concentrations on the basis of active transport, being transported to cells in oxidized form, as dehydroascorbic acid (DHA), with the participation of glucose [23]. It circulates freely, reaching all tissues and cells, and therefore occurs both in plasma and inside cells (especially in leukocytes and platelets). The highest concentrations of ascorbic acid have been observed in glandular tissues, and the lowest in muscle and adipose tissue.

Within cells, dehydroascorbic acid is reduced to ascorbate and stored in this form. Ascorbic acid is mainly metabolized to oxalate excreted in the urine. Ascorbic acid excretion is controlled by the so-called renal threshold, 1.5 mg/100 ml. Exceeding this threshold in the plasma leads to an increased excretion of ascorbic acid with urine in unchanged form, as ascorbic acid, and can also be excreted as dehydroascorbic acid. The absorption of ascorbic acid significantly depends on the presence of biotin and folic acid.

The reserves of ascorbic acid in the body are sufficient for 3-4 months, as the rate of catabolism is about 3% per day. Increased demand for ascorbic acid occurs in athletes, in people staying in cold or in hot weather, and during diseases. Deficiency is commonly observed in breast-fed infants, undernourished people, dialysis patients [29], during chronic diseases (tuberculosis, rheumatic disease), alcohol drinkers and smokers [20, 26]. For women, in addition to frequently occurring qualitative malnutrition, the level of supply with ascorbic acid may also be significantly affected by oral contraceptives that may affect the metabolism of ascorbic acid and cause an increased risk of cervical dysplasia [8]. Ascorbic acid excess in the diet is relatively rarely recorded, and may cause formation of kidney oxalate stones. However, the formation of oxalate stones relates to the consumption of extremely high doses of L-ascorbic acid. Some of the products may also contain ascorbic acid that especially in combination with benzoic acid can cause exposure to benzene [11]. A much greater threat is associated with ascorbic acid deficiency, because as a highly reactive antioxidant it participates in many reactions responsible for disposing of free radicals. Vitamin C supplementation is indicated in individuals exposed to free radicals (ROS), generated during pathogenic processes, among smokers [19] and athletes. The effect of vitamin C supplementation is most noticeable 2-8 h after consumption [22]. It is interesting to note that despite ascorbic acid antioxidant function, no links have been found between levels of this vitamin and the incidence of prostate cancer [1]. Currently, recommended in Poland daily intakes of vitamins C for women are 60 mg (EAR level) and 75 mg (RDA level) and for men 75 and 90 mg per day, respectively [3]. However, some the researchers express the opinion that these standards may be too low and inadequate for adults as compared to the recommended dietary intake of vitamin C in Western Europe and North America (75-90 mg per day) [9]. Moreover, taking into account that smoking increases oxidative stress and metabolic turnover of vitamin C, the requirement for smokers is increased by 35 mg/day. And the average daily intake of vitamin C in the Mediterranean countries is significantly higher and amounts to more than 140 in Greece per day [16].

The aim of this paper was to study a correlation between composition of nutrients in daily diet and plasma vitamin C levels in university students. This study was undertaken in relation to the fact that despite frequent supplementation (being the most popular in the examined population), the quantity of vitamin C absorbed from enriched foods and dietary supplements in daily food rations is rarely examined.

MATERIAL AND METHODS

Examined group

The experiments were conducted on a group of students from the Department of Food Technology and Human Nutrition at the West Pomeranian University of Technology in Szczecin. Prior to experiments the consent of the Bioethical Commission at the Pomeranian University of Medical (Declaration of Helsinki) was obtained [2]. Smoking and non-smoking students (96 women and 24 men) voluntarily took part in the research program. Disproportion between the sexes resulted from the type of the studies, where the majority of students are female. The age of the subjects ranged from 22 to 25. Nutritional status was assessed using anthropometric methods with Body Mass Index (BMI) and Waist to Hip Ratio (WHR). The interview with the participants, concerning their health status, symptoms characteristic for ascorbic acid deficiency, medical treatment, diet supplements, smoking and oral contraception, was carried out on the day of blood collection.

Dietary assessment method

The examination was performed in February. Diets were assessed by 7-days food report method. After that blood was taken from all participants, centrifuged (4˚C/10 min/2500 g/dark) and plasma was collected in -80˚C to future analysis. 7-days food reports included information on the supply of dietary supplements and foods enriched were collected. Daily diet were identified using the "Album of photographs of food products and dishes" [25]. The content of selected nutrients in daily diet such as: energy, proteins, lipids, carbohydrates, vitamins A, D, E, C, B1, B2, B6, B12, niacin, folate, and Na, K, Ca, P , Mg, Fe, Cu, Zn, Mn, fibre and cholesterol was calculated using the computer program "Dietetyk 2" (National Food and Nutrition Institute, Warsaw, Poland) taking nutrient loss into account [13].

Statistical analysis

Arithmetic mean (x), standard deviation (SD) and the significance of differences at $p \leq 0.05$ were calculated using a statistical software package Statistica version 8.0 (Statsoft, Tulsa, Oklahoma, United States).

Determination of ascorbic acid by HPLC method

Blood was collected in tubes (Sarsted) containing EDTA anticoagulant. The samples were centrifuged for 10 min at 2500 rpm at 4°C. Plasma from centrifuged blood was collected into Merck amber Eppendorf safe lock containers and stored at -80°C. All the samples were determined and stored in signed containers. 1 month after collection using the procedure described above plasma was thawed and vortexed. Ascorbic acid was determined by HPLC. After adding equal amounts of EDTA and HPO_3 solution, the samples were centrifuged for 10 min at 2°C. The final stage was the injection of the supernatant and measurement at 243 nm in Hewlett Packard liquid chromatograph. The measurement was duplicated. Quantitative determination was based on the peak area for internal standard [17]. Normal status ascorbic acid nutrition was assumed at the level above 55 µmol/L in plasma.

RESULTS

Interview and general examinations

The students were a random group. Women had a mean BMI of 21.2 and men 23.4. The average value of WHR among all women was 0.79 and men 0.93. As a result of general examination, five persons were noted as during antibiotic-therapy or shortly (up to 2 weeks) after such treatment. Oral hormonal contraception was used by an average of 34.4% of female students, while 28.1% of women and 41.6% of men were smokers. Clinical symptoms that may indicate a deficit of ascorbic acid were reported by 41.7% of women and 54.2% of men (bleeding from the gums, decrease in immune response, dry skin on knees and elbows, papules on the forearms and thighs). Because symptoms are nonspecific, we toke taken into account people who identified 3 of the 4 symptoms. In the examined population 24.6% of the students reported supplementation with vitamin C during 7 days prior to collection of blood samples. The average amount ingested ascorbic acid among women and men was respectively 66 and 60 mg/day/person. In the study group there were no people at risk of exceeding vitamin C upper tolerable level (UL). It seems that the often overlooked sources of ascorbic acid can significantly affect its status in the human body.

Table 1. Population characteristics of selected essential features of the nutritional status of vitamin C

Descriptor	Women n=96	Men n=24	Population n=120
% of students with clinical symptoms that may indicate a deficit of ascorbic acid	41.7	54.2	44.2
% of students with oral hormonal contraception used	34.4	-	-
% of students reported smoking	28.1	41.6	30.8
% of students with ascorbic acid concentration < 11 µmol/L	1.1	0.0	0.8
% of students with ascorbic acid concentration 11-28 µmol/L	5.2	12.5	6.7
% of students with ascorbic acid concentration 29-39,9 µmol/L	18.7	16.7	18.3
% of students with ascorbic acid concentration 40-55 µmol/L	50.0	45.8	49.1
% of students with ascorbic acid concentration >55 µmol/L	25.0	25.0	25.0
the average concentration of ascorbic acid in plasma [µmol/L]	48.65	45.61	48.04
the average concentration of ascorbic acid in plasma <40 µmol/L	32.2	29.6	30.0
the average concentration of ascorbic acid in plasma 40-55 µmol/L	47.63	46.97	47.5
the average concentration of ascorbic acid in plasma >55 µmol/L	65.12	64.77	64.99
The average daily intake with supplementation of vitamin C (mg). The group ascorbic acid in plasma <40 µmol/L	46.55	48.56	47.03
The average daily intake with supplementation of vitamin C (mg). The group ascorbic acid in plasma 40-55 µmol/L	46.74	45.55	46.5
The average daily intake with supplementation of vitamin C (mg). The group ascorbic acid in plasma >55 µmol/L	62.06	66.26	62.76

Provision with ascorbic acid

Ascorbic acid nutritional status in the majority of examined students was correct (94.8% women and 87.5% men) (Table 1). Biochemical evidence of marginal ascorbic acid concentration in plasma (11-28 μmol/L) was observed in 6.7% of the population. Biochemical symptom of ascorbic acid deficiency (less than 11 μmol/L) were observed in only one person. The average concentration of ascorbic acid in plasma in women amounted to 48.65 μmol/L and in men 45.61 μmol/L (Table 1). Assessment of significant differences in ascorbic acid provision between the sexes did not show better provision in any of the groups. Comparison of plasma concentrations of ascorbic acid content of the 7-day diet record have showed an average ascorbic acid intake of 47.03 mg for those at risk of ascorbic acid deficiency (concentration <40 μmol/L). People who were not at risk of ascorbic acid deficiency (concentration >55 μmol/L), consumed an average of 62.76 mg of this vitamin in daily diet (Table 1).

Daily diet (DD) with respect to concentration ascorbic acid in plasma

DD composition analysis in relation to the concentration of ascorbic acid in plasma showed that in the group, with the highest concentration of ascorbic acid in plasma (group III), both in women and men, diets were richer in nutrients (in respectively 13 and 16 tested ingredients, Table 2) than group II and group I in women. Who consumed a similar value of energy as group III, but energy was provided by disaccharides (saccharose) to a larger extent than group II. Differences in the nutrient content of DD of men were even more pronounced (Table 3). In the group of men with the lowest concentration of ascorbic acid in plasma (group I) DD was significantly richer in energy than macronutrients (protein, lipids, carbohydrates) compared with group II (ascorbic acid concentration in plasma 40-55 μmol/L). Moreover, the intake of saturated fatty acids was significantly higher and the intake of polyunsaturated fatty acids was significantly lower than group

Table 2. Energy and nutrients content in daily diet in women and the ascorbic acid status in plasma (μmol/L)

Discriminant	Plasma ascorbic acid concentration ranges		
	Group I <40 n=22	Group II 40-55 n=50	Group III >55 n=24
Energy [kcal]	1742.6^{ab} ± 324.4	1688.4^{a} ± 310.7	1885.6^{b} ± 442.3
Protein [g]	62.9 ^{a} ± 12.0	61.1 ^{a} ± 12.3	64.0^{a} ± 12.9
Lipids [g]	69.0 ^{a} ± 18.1	66.6 ^{a} ± 14.7	73.3 ^{a} ± 20.3
SUFA [g]	26.0 ^{a} ± 7.4	24.2 ^{a} ± 5.6	26.2 ^{a} ± 7.7
MUFA[g]	27.5 ^{a} ± 7.9	26.7 ^{a} ± 6.6	29.8 ^{a} ± 9.3
PUFA [g]	10.2 ^{a} ± 3.1	10.5 ^{a} ± 3.7	11.8 ^{a} ± 3.7
Cholesterol [mg]	245.5 ^{a} ± 89.9	233.5 ^{a} ± 79.6	226.2 ^{a} ± 64.1
Carbohydrates [g]	225.8^{a} ± 48.7	221.7^{a} ± 44.4	252.1^{b} ± 64.0
Saccharose [g]	57.4^{ab} ± 21.8	54.6^{a} ± 20.2	66.3^{b} ± 30.3
Lactose [g]	8.8 ^{a} ± 3.6	7.5 ^{a} ± 3.6	9.4 ^{a} ± 4.4
Dietary fibre [g]	14.2^{a} ± 3.3	15.6^{a} ± 3.7	17.8^{b} ± 4.6
Na [mg]	1697.5 ^{a} ± 400.1	1782.8 ^{a} ± 423.5	1777.8 ^{a} ± 454.5
K [mg]	2286.0^{a} ± 428.5	2332.5^{a} ± 476.3	2694.5^{b} ± 694.4
Ca [mg]	598.9 ^{a} ± 158.9	552.9 ^{a} ± 155.4	594.5 ^{a} ± 208.5
P [mg]	1028.2 ^{a} ± 184.0	1010.2 ^{a} ± 189.2	1078.0 ^{a} ± 249.5
Mg [mg]	236.6^{a} ± 48.8	230.7^{a} ± 51.6	266.3^{b} ± 67.1
Fe [mg]	8.83^{a} ± 1.58	8.79^{a} ± 2.40	10.38^{b} ± 3.70
Zn [mg]	8.23 ^{a} ± 1.54	7.99 ^{a} ± 1.57	8.96 ^{a} ± 3.69
Cu [mg]	0.93^{a} ± 0.19	0.94^{a} ± 0.22	1.23^{b} ± 0.52
Mn [mg]	3.69 ^{a} ± 0.73	3.72 ^{a} ± 1.01	4.23 ^{a} ± 1.47
Vitamin A [μg]	694.2 ^{a} ± 279.6	962.0 ^{a} ± 1180	931.9 ^{a} ± 576.6
Vitamin D [μg]	2.80 ^{a} ± 1.78	2.56 ^{a} ± 1.50	2.74 ^{a} ± 1.49
Vitamin E [mg]	7.40^{a} ± 2.79	7.56^{a} ± 2.74	9.16^{b} ± 3.55
Vitamin B1 [mg]	0.88 ^{a} ± 0.21	0.90 ^{a} ± 0.27	0.93 ^{a} ± 0.21
Vitamin B2 [mg]	1.25 ^{a} ± 0.24	1.26 ^{a} ± 0.36	1.36 ^{a} ± 0.35
Niacin [mg]	12.59^{a} ± 3.69	13.44^{ab} ± 4.59	15.63^{b} ± 9.35
Vitamin B6 [mg]	1.36^{a} ± 0.29	1.47^{a} ± 0.46	1.68^{b} ± 0.53
Folacin [μg]	142.83^{a} ± 30.40	145.25^{a} ± 38.23	178.75^{b} ± 96.82
Vitamin B12[μg]	3.30 ^{a} ± 2.11	3.37 ^{a} ± 2.83	3.60 ^{a} ± 1.98
Vitamin C [mg]	46.55^{a} ± 48.37	46.74^{a} ± 34.27	62.06^{b} ± 62.15

^{a,b}homogeneous groups according to the *Tuckey* test

Table 3 Content of nutrients in daily diet in men and the ascorbic acid status in plasma (μmol/L)

Discriminant	Plasma ascorbic acid concentration ranges		
	Group I <40 n=7	Group II 40-55 n=11	Group III >55 n=6
Energy [kcal]	2444.0[b] ± 490.7	2115.3[a] ± 404.6	2428.6[b] ± 506.1
Protein [g]	83.2[b] ± 15.7	76.0[a] ± 14.0	85.4[b] ± 14.2
Lipids [g]	100.7[b] ± 25.4	86.3[a] ± 22.4	101.1[b] ± 20.5
SUFA [g]	37.1[b] ± 12.6	31.7[a] ± 9.8	36.2[b] ± 7.8
MUFA[g]	41.0 ± 10.5	35.1[a] ± 9.8	39.7[a] ± 9.8
PUFA [g]	14.8[a] ± 2.4	13.0[a] ± 4.1	17.7[b] ± 4.3
Cholesterol [mg]	344.3[a] ± 151.7	286.5[a] ± 94.9	320.1[a] ± 76.3
Carbohydrates [g]	309.8[b] ± 68.2	275.7[a] ± 54.9	315.1[b] ± 76.2
Saccharose [g]	59.6[a] ± 10.5	61.2[a] ± 23.1	80.3[b] ± 19.1
Lactose [g]	13.0[a] ± 7.5	10.8[a] ± 6.0	11.3[a] ± 5.4
Dietary fibre [g]	20.0[a] ± 4.6	18.9[a] ± 5.4	22.6[b] ± 8.9
Na [mg]	2609.9[a] ± 754.0	2194.8[a] ± 635.1	2500.8[a] ± 364.4
K [mg]	2938.9[a] ± 686.0	2811.7[a] ± 510.2	3378.9[b] ± 1029.8
Ca [mg]	725.2[a] ± 223.4	706.0[a] ± 250.3	832.8[a] ± 238.1
P [mg]	1382.9[ab] ± 266.0	1255.6[a] ± 293.0	1473.4[b] ± 261.1
Mg [mg]	302.9[ab] ± 59.1	275.1[a] ± 73.0	337.9[b] ± 108.0
Fe [mg]	10.96[a] ± 2.19	11.91[ab] ± 2.79	12.42[b] ± 2.94
Zn [mg]	11.03[ab] ± 1.84	10.30[a] ± 2.42	11.93[b] ± 1.74
Cu [mg]	1.15[a] ± 0.19	1.12[a] ± 0.27	1.45[b] ± 0.49
Mn [mg]	4.69[a] ± 1.33	4.77[a] ± 1.89	5.61[a] ± 2.26
Vitamin A [μg]	990.38[a] ± 764.85	843.11[a] ± 381.92	1186.46[a] ± 654.42
Vitamin D [μg]	3.83[a] ± 1.68	3.07[a] ± 1.22	4.14[a] ± 2.12
Vitamin E [mg]	9.83[a] ± 1.63	8.77[a] ± 3.08	12.47[b] ± 3.13
Vitamin B1 [mg]	1.34[a] ± 0.26	1.30[a] ± 0.30	1.28[a] ± 0.52
Vitamin B2 [mg]	1.76[a] ± 0.46	1.61[a] ± 0.35	1.64[a] ± 0.32
Niacin [mg]	17.22[a] ± 5.64	16.94[a] ± 3.41	15.75[a] ± 4.33
Vitamin B6 [mg]	1.95[a] ± 0.57	1.97[a] ± 0.38	1.94[a] ± 0.58
Folacin [μg]	188.75[a] ± 65.56	172.33[a] ± 38.56	185.76[a] ± 53.44
Vitamin B12 [μg]	5.75[b] ± 3.55	3.48[a] ± 1.47	3.88[a] ± 0.63
Vitamin C [mg]	48.56[a] ± 30.79	45.55[a] ± 15.69	66.26[b] ± 10.38

[a,b]-homogeneous groups according to the *Tuckey* test

III. The intake of phosphorus, magnesium and zinc did not differ in the compared extreme groups (I and III). The intake of vitamin E and C and minerals such as potassium, iron and copper was significantly lower than group III (Table 3).

Correlation between diet and ascorbic acid contents

Table 4 show the relationship between the content of analyzed nutrients in diets and ascorbic acid contents in daily diet with reference to sexes. In the female subjects, the nutrients of the greatest importance were (in order of significance): dietary fiber, potassium, calcium, phosphor, magnesium, iron and folate (correlation significance 0.3 – 0.2). In the male group the corresponding energy, cholesterol and nutrients were: protein, lipids, MUFA, PUFA, carbohydrates, sodium, calcium, phosphorus, iron, zinc, vitamin E, folate (correlation significance 0.62 – 0.40). This suggests that the major source of ascorbic acid in DD of female students was greater consumption of fruit and vegetable in daily diet.

DISCUSSION

The most frequently reported indicator of ascorbic acid status in the human body is blood plasma concentration, considered to be correct when in the range 60 μmol/L to 180 μmol/L [18]. However, as suggested by *Lykkesfeld* [15], the optimal concentration of ascorbic acid in plasma should exceed 70 μmol/L. A decline in ascorbic acid concentration below 28 μmol/L shows a marginal deficiency of ascorbic acid. Concentrations below 11 μmol/L (0.2 mg/100 ml) were classified as biochemical evidence and/or clinical symptoms of scurvy [10]. The survey carried out in the current work shows that the concentration of ascorbic acid in plasma that demonstrates a marginal deficiency should be at least 40 μmol/L (and even least 55 μmol/L). As dietary consumption of vitamin C in these subjects was only about 45 mg per day.

It is assumed that the daily intake of vitamin C for adults amounting to 60-75 mg is sufficient to maintain

Table 4 Correlation between diet and ascorbic acid content in female and male groups

Discriminant	Female	Male
Energy [kcal]	0.54	0.16
Protein [g]	0.43	0.18
Lipids [g]	0.46	-0.03
SUFA [g]	0.35	-0.01
MUFA[g]	0.45	-0.05
PUFA [g]	0.4	-0.01
Cholesterol [mg]	0.43	-0.12
Carbohydrates [g]	0.42	0.19
Saccharose [g]	0.31	0.07
Lactose [g]	-0.03	0.08
Dietary fibre [g]	0.31	0.26
Na [mg]	0.43	0.12
K [mg]	0.36	0.3
Ca [mg]	0.44	0.23
P [mg]	0.42	0.22
Mg [mg]	0.32	0.3
Fe [mg]	0.46	0.2
Zn [mg]	0.43	0.09
Cu [mg]	0.31	0.15
Mn [mg]	0.22	0.14
Vitamin A	0.26	0.02
Vitamin D	0.25	0.13
Vitamin E	0.46	0.04
Vitamin B1	0.30	0.16
Vitamin B2	0.36	0.12
Niacin	0.18	0.19
Vitamin B6	0.21	0.18
Folacin	0.62	0.28
Vitamin B12	0.07	-0.09
Vitamin C	1.0	1.0

correlation significance for female r>0.2; correlation significance for male r>0.38

an adequate level in plasma and sufficient leukocyte saturation [18]. However, when comparing the average intake of this vitamin among the Greek the level at 140 mg, among the Japanese at 115 mg [7, 10, 16, 24] it would be prudent to consider an increase in daily ascorbic acid by Poles. In nearly a quarter of the study group, even the use of ascorbic acid supplements at around 60mg/day/person did not significantly change the trend of low concentrations of ascorbic acid in plasma.

Importantly, the level of ascorbic acid in the blood is affected not only by the diet (including the treatment of products) and possible supplementation but also factors not related to diet, such as health status, physical activity, drinking alcohol and smoking tobacco. In assessing the body's supply with vitamin C in young athletes it was found that physical exercise causes a decrease of serum in serum by about 15%, while supplementation significantly improve the players' physical fitness tests [4]. Controversial results by *Chawla* [4], exist which show that fitness results depended on vitamin E concentration in plasma and not ascorbic acid.

It seems that the cause of the adequate supply of vitamin C in Poland is the insufficient consumption of fruit and vegetables, in winter. This is due to high prices or irregular eating habits and easy access to processed foods. Troubling results for the too low intake of ascorbic acid found in the younger group of Polish population. The vitamin C intake in daily diets of girls and boys aged 13-18 was deficient [6]. Studies from *Leszczynska* confirm low intake of vitamin C in students (30.0 and 35.4 mg per day) [14]. Considerable variation in the consumption of vitamin C in daily food rations using advanced statistical analysis methods showed *Wądołowska* [28].

The trend of lower intake of vitamin C in winter was observed in students from Arizona [12], with marginal intake of vitamin C observed in 11% students in autumn and in 16% in winter (below 28 μmol/L in plasma). In contrast, vitamin C status among Indians after one month stay in the Antarctic decreased from an initial level of 1.31 to 0.81 mg/dL (74,4-45,9 μmol/L), which undoubtedly shows the influence of season on the nutritional status of the human body [27].

Comparing the average concentrations of vitamin C levels among students from different places around the globe, it should be noted that among students of Arizona the average concentration was 44.7 and 41.5 μmol/L [12], higher levels of vitamin C in plasma (54 and 62 μmol/L) were observed among students from Florida [21]. The highest concentration of vitamin C (62 and 67 μmol/L) was found in Japanese students diets rich in plant food [24].

The risk of scurvy (ascorbic acid <11 μmol/L) in a study by *Johnston* et al. [12] was measured 1-2% of the population, while among Polish students in our study it was measured only one person (0.8% of population). This could be the result of either a larger supply of ascorbic acid or - seemingly more likely - differences in the methodology of plasma sample preparation. In our study samples were strictly handled, i.e. within 15 min of collection they had been centrifuged, separated, stored in a dark room and frozen in amber Eppendorf, which certainly had a positive effect on reducing the losses of ascorbic acid in the sample. This supposition is supported by the fact that concentration of ascorbic acid in Polish populations, studied by other authors, was relatively lower. It should be noted that the average concentrations of ascorbic acid in the blood of Polish students (48.6 μmol/L in women and 45.6 μmol/L in men) did not differ from results obtained in the group of students from Arizona, but were lower than students of both sexes from Florida, and students from Japan. The percentage of the population having ascorbic acid concentrations considered marginal (12-28 μmol/l) was 6.2%.

CONCLUSIONS

1. It seems that the range of marginal ascorbic acid concentrations in plasma should be increased from 12 to 40 µmol/L and even to 55 µmol/L, because the intake of vitamin C in these fields in the plasma was significantly lower.
2. In one quarter of the study group the dietary supplementation with ascorbic acid did not cause increased ascorbic acid levels in the plasma.
3. Despite low intake of vitamin C, the risk of serious deficiency among respondent students is very low.

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Conflict of interest

The authors declare no conflict of interest.

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COMPARING OF NUTRIENTS CONTENT AND CALORIFIC VALUE IN THE DIETS OF POLES AND GREEKS LIVING IN ATHENS

Monika Bronkowska[*]*, Martyna Wtykło, Ewa Bator, Dagmara Orzeł, Jadwiga Biernat*

Department of Human Nutrition, Faculty of Food Science, Wroclaw University of Environmental and Life Sciences, Wroclaw Poland

ABSTRACT

Background. Diet is generally defined as a set of behaviors, on the choice of certain foods, their consumption, and it is conditioned by various factors. Each model is shaped feeding in a given place and time by repeated regularly eating and dietary habits. Polish migration to Greece contributed not only to change their place of residence, but also forced them to adapt to a new country. In the literature, foreign language is used for this purpose, the concept of acculturation, which can be distinguished in many aspects of life as well as on the diet. Acculturation related to a diet is called acculturation nutritional and can cause desirable or undesirable effects on nutrition.

Objective. To compare calorific intakes and core nutritional components in the daily diets of Poles and native Greeks, residing in Athens.

Materials and methods. Three repeats of completing a 24-hour food questionnaire were performed for the study. Results were compared with known nutritional requirements and recommendations (according to weighted means). *Subjects:* These were recruited at the turn of 2010/2011 in Athens and consisted of 66 persons aged 19-26, 31-50 and 51-65 years of whom 31 were Polish and 35 Greek. In the former, 18 (58%) were women and 13 (42%) men, whilst in the latter 19 were women (54%) and 16 men (46%).

Results. Daily dietary intakes of calories were 1832 kcal for the Poles but 1628 kcal for the Greeks. Significant differences were observed between the subject groups for daily dietary intakes of calories, total carbohydrate, fibre, saturated fatty acids (SFA) and poly-unsaturated fatty acids (PUFA). In women subjects, both Polish and Greek, these significant differences were seen in calorific and carbohydrate intakes, whereas for Polish and Greek men such differences were limited only to dietary fibre.

Conclusions. The daily diets of Poles and Greek subjects living in Athens did not meet recommended standards of proper nourishment.

Key words: *nutrients, calories, daily dietary intakes, Poles, Greeks, Athens, saturated fatty acids, SFA, poly-unsaturated fatty acids, PUFA*

STRESZCZENIE

Wprowadzenie. Sposób żywienia jest na ogół definiowany jako zbiór zachowań, dotyczących wyboru określonych produktów spożywczych, ich konsumpcji i jest on uwarunkowany różnymi czynnikami. Każdy model żywienia kształtowany jest w danym miejscu i czasie przez powtarzające się regularnie nawyki oraz zwyczaje żywieniowe. Migracja Polaków do Grecji przyczyniła się nie tylko do zmiany ich miejsca zamieszkania, ale również zmusiła ich do adaptacji w nowym kraju. W literaturze obcojęzycznej używa się do tego celu pojęcia akulturacji, które może być rozróżniane w wielu aspektach życia, również dotyczących diety. Akulturacja związana z dietą nazywana jest akulturacją żywieniową i może wywołać pożądane, bądź niepożądane skutki w odżywianiu.

Cel. Celem pracy było porównanie podaży energii i składników podstawowych w całodziennych racjach pokarmowych Polaków mieszkających w Grecji, jak i rdzennych Greków, mieszkańców Aten.

Materiał i metody. Przeprowadzono wywiady żywieniowe z ostatnich 24 godzin przed badaniem, powtórzone trzykrotnie. Wyniki porównano z wymaganiami i zaleceniami żywieniowymi. W badaniach wykonanych w latach 2010/2011 w Atenach uczestniczyło 66 osób w wieku 19-26, 31-50 and 51-65 lat, z których 31 stanowili Polacy i 35 Grecy.

Wyniki. W całodziennych racjach pokarmowych Polaków podaż energii wynosiła 1832 kcal, natomiast Greków - 1628 kcal. Nie stwierdzono istotnych różnic statystycznych w całodziennych racjach pokarmowych Polaków (n = 31) i Greków (n = 35), w zakresie podaży energii, węglowodanów ogółem, błonnika, SFA oraz PUFA. Jeśli chodzi o całodzienne racje pokar-

*** Corresponding author**: Monika Bronkowska, The Faculty of Food Science, Department of Human Nutrition, Wroclaw University of Environmental and Life Sciences, Chełmońskiego 37/41, 51-630 Wroclaw; phone: +48 71 320 7715, e-mail: monika.bronkowska@wnoz.up.wroc.pl

mowe polskich (n = 18) i greckich (n = 19) kobiet to nie zaobserwowano istotnych różnic w podaży energii i węglowodanów ogółem, podczas gdy wśród polskich (n = 31) i greckich (n = 16) mężczyzn stwierdzono istotne różnice w podaży błonnika. **Wnioski.** Całodzienne racje pokarmowe zarówno osób pochodzenia polskiego, mieszkających w Grecji jak i greckiego – mieszkańców Aten nie realizowały przyjętych norm i zaleceń prawidłowego odżywiania.

Słowa kluczowe: *składniki odżywcze, energia, dzienne racje pokarmowe, Polacy, Grecy, Ateny, błonnik, węglowodany, nasycone kwasy tłuszczowe, SFA, wielonienasycone kwasy tłuszczowe, PUFA*

INTRODUCTION

Migration throughout Europe is a widespread phenomenon. When migrants change their country of residence, changes in dietary habits often concomitantly happen, that so bear a direct effect their health [10]. Poles living in Greece are one of the oldest and one of the ten largest immigration groups in Greece. Currently, the Polish Embassy in Athens estimates that there are around 30,000 to 35,000 Poles living in Greece [6]. Information on the inhabitant's nutritional preferences can therefore be essential to food producers, dieticians and nutrition experts.

Many studies have demonstrated that the risk of heart disease, cancer, *Parkinson's* and *Alzheimer's* disease are decreased in those living around and adopting the traditional Mediterranean Sea diet [1]. The first results were published by *Keys* whose thesis was subsequently confirmed by many other studies. It was additionally proved that this 'Mediterranean diet' (MD) also has beneficial effects on fat metabolism, blood pressure, blood coagulability and the BMI [7]. Its pro-healthy features arise from a high intake of vegetable foodstuffs, but low intakes of animal products. The main components of the MD are cereal products from whole grains, vegetables, fruit (3 portions per day), and pulses and legumes (3-4 weekly portions). Dairy products are consumed twice daily, whereas fish and seafood 5-6 times weekly, with red meat and its products 4 times per month. The high olive oil intake, which is typical in these regions, was regarded as the valuable source of monounsaturated fatty acids (MUFA) and vitamin E. Alcohol intake was limited to 1-2 glasses of red wine per day. The MD diet was consequently low in calories but rich in macrocomponents, vitamins and mineral compounds and, as such, it has played an important role in preventing cardiovascular system disease. This way of nutrition has been characteristic in the inhabitants of villages and country towns, whereas in cities the so called 'western' diet, that is rich in fat, is starting to predominate.

The study aim was to compare the dietary intakes of calories and core components for Poles and native Greeks living in Athens.

MATERIALS AND METHODS

The study was performed at the turn of 2010/2011 in Athens among 66 people of whom 31 were Polish and 35 Greek.

A 24-hr food questionnaire, repeated three times, was completed by both these subject groups. The sizes of consumed portions were assessed on the basis of an '*Album of products and meals portions*' [12], from which calorific values and core nutrients' content in the daily diet were calculated. To this end, the *Energia* v. 4.1 computer programme was used with the '*Tables of chemical composition and nutritional value of foodstuffs*' [5], providing the database. The programme allowed for 10% losses arising from food processing. Results were compared with standards and recommendations (according to weighted mean) and calculated by the following formula:

$$Z = \frac{S_1U_1 + S_2U_2 + ... + S_xU_x}{100}$$

where:
Z = weighted mean of nutrient demand per person,
$S_1S_2...S_x$ = calorific demand for each member from 'x' group,
$U_1U_2...U_x$ = percentage share of a given groups' participation.

Standards and recommendations used for the calculations have been earlier described by *Jarosz* and *Bułhak- Jahymczyk* [2] based on the average demand – EAR. On account of the large differences in standards and recommendations (related to age and gender) as well as to simplify the calculations, female and male subjects were divided into the following age groups; 19-30, 31-50 and 51-65 years (Table 1). The weighted mean of the calorific demands as calculated by the above formula were 2012 kcal for women and 2878.4 kcal for men.

Table 1. Demographic characteristics of Polish and Greek subjects participated in the study

Poles and Greeks				
Age	Women		Men	
	n=37	% of group	n=29	% of group
19-30	22	59%	22	76%
31-50	13	35%	6	21%
51-65	2	5%	1	3%

Using the STATISTICA PL, in v. 9.0 by StatSoft programme, statistical analysis was performed allowing a comparison of calorific and macronutrients intakes to be made between the Polish and Greek diets. The test used was the non-parametric *Mann-Whitney* U, adopting a $p \leq 0.05$ level for significance.

RESULTS

Among the 31 Poles 18 (58%) were women and 13 (42%) men, whilst for the 35 Greeks, 19 were women (54%) and 16 men (46%). Compared with the Greeks, Poles were more physically active where 15 subjects (48%) declared an average level and 10 (32%) had high levels of physical activity. Most of the Greeks (16 ie. 46%) were not physically active. The majority in both groups were young people, aged 19-30 years (64.5% Poles, 68.6% Greeks). The average age of the Poles was 28.7 years for women and 29 years for men, but in Greeks these were 31.7 years for women and 28 years for men. The results of the studies are presented in tables 2-4.

Table 2. The energy and nutrients supply in daily food rations of Poles (n= 31) and Greeks (n= 35)

Paramaters	Unit	Average value of analyzed variable		U *Mann-Whitney* test p
		Poles	Greeks	
Energetic value	kcal	1832	1628	0.02*
Total protein	g	69.9	66.0	0.31
Animal protein	g	46.5	45.3	0,67
Vegetable protein	g	23.2	20,7	0.12
Total fat	g	69.9	84.5	0.10
Total carbohydrates	g	224.9	195.1	0.04*
Fiber	g	18.1	14.8	0.03*
SFA	g	28.0	24.7	0.006*
MUFA	g	31.3	29.7	0.39
PUFA	g	11.7	9.5	0.006*
Cholesterol	mg	270.5	239.9	0.14

*differences statistically significant at $p < 0.05$

The daily dietary calorific intakes for Poles was 1832 kcal but 1628 kcal for the Greeks. In both groups, the calorific intakes were lower than the calculated demand. Polish women met this demand at 84%, whereas Greek females at 73%. Polish men achieved the calorific EAR standard at 71%, whereas the Greeks at 63%. Dietary calorific intakes were higher in Polish women than Greek women; respectively 1680.1 kcal, (ie. 84% of recommendations) vs. 1466.0 kcal (ie. 73%). Likewise in men, the Poles' calorific intakes were higher than in Greeks; respectively on average 2041.8 kcal (ie. 71% of recommendations), vs. 1819.6 kcal (ie. 63%).

Total daily intakes of dietary protein was on average 69.9 g (range: 39–129 g) for the Poles and 66.0 g (range: 41.7-113.6 g) for the Greeks. According to the calculated weighted mean, the EAR recommended protein allowance for women should be 58.8 g, and 77.2 g for men. On average, the dietary daily intake of protein in Polish women was 62.6 g (107% of the standard), out of which 41.6 g, (212% of the recommendations), was of animal origin and 21.0 g, (54% of the recommendations), was vegetable derived protein. Greek women consumed 58.9 g of this nutrient and thus properly fulfilled standard at 100%. The intake types of protein were however unsuitable in both Greek and Polish women where about 40.6 g (207% of the recommendations) was of animal origin, but only 18.3 g (47% of the recommendations) were vegetable derived. In Polish men the protein intake was 79.4 g (ie. 103% of the recommendations), of which 53.2 g (207% of the recommendations) was animal protein, but only 26.2 g (51% of recommendation) was vegetable derived. Greek protein intakes were 74.5 g (97% of the recommendations) for men, of which 50.9 g (198% of the recommendations) was animal protein, but only 23.6 g (46% of the recommendations) came from vegetables. Summing up, respectively 38.9% and 57.9% of Polish and Greek women along with 61.5% and 62.5% of Polish and Greek males fulfilled dietary recommendations for consuming vegetable protein within the 30-50% range.

The subjects' diets met the demand by more than 110% to the recommended levels. Despite the fact that the ratio of vegetable to animal protein in a healthy adult diet should be 2:1, none of the surveyed groups' meals fulfilled this particular condition.

The Poles' daily diet delivered an average 224.9 g of carbohydrates (range: 95-334 g), whereas the Greeks yielded 195.1 g (range: 110.1-360.2 g). According to the EAR standard, calculated as the weighted mean, the daily dietary intake should however provide women with 276.6 g of carbohydrates and men with 395.8 g. It was therefore observed that both these groups consumed too little of this nutrient. In detail, Polish women received 211.6 g of carbohydrates in their diet, accounting for 77% of recommended standard. In Greek women, this was even less at 172.9 g (63% of the standard) of dietary carbohydrate. Carbohydrate intakes for Polish men was 243.2 g (61% of the recommended standard), whereas the Greeks' daily diet delivered 221.4 g of carbohydrates (56% of the recommended standard).

According to the established recommendations, the amount of fibre necessary in a daily diet should be at least 30 grams per day. In general, the Poles' daily meals were richer in this nutrient than for Greeks, being 18.1 g (range: 8.7 – 33.2 g) for the former and 14.8 g for the latter (range: 7.2 – 27.3 g). Daily dietary fibre intakes in Polish women were 15.9 g (53% of the recommendations), whereas they were 13.6 g (45% of the recommendations) for Greek women. Polish men consumed 21 g of dietary fibre (70% of the recommendations), whereas

Table 3. The average supply of energetic value and core nutrients in investigated group of Polish (n=18) and Greek (n=19) women

Nutrients	Unit	Average supply		Standard deviation (±SD)		Minimum		Maximum		Median		Standards and recommendations – weighted mean	% of standards realisation	
		Polish women	Greek women	Polish women	Greek women	Polish women	Greek women	Polish women	Greek women	Polish women	Greek women		Polish women	Greek women
energetic value*	kcal	1680.1	1466.0	359.9	353.7	1000.0	1006.0	2275.0	2181.0	1683.5	1344.0	2012.0	84	73
total protein	g	62.6	58.9	13.0	13.9	39.0	41.7	82.3	96.0	62.4	54.0	58.8	107	100
animal protein	g	41.6	40.6	9.1	11.8	27.7	24.8	62.9	65.4	40.6	37.8	19.6	212	207
vegetable protein	g	21.0	18.3	6.1	6.2	10.6	9.8	34.6	35.7	20.0	16.0	39.2	54	47
total fat	g	69.9	63.1	15.1	16.4	40.3	27.4	98.4	109.5	66.4	60.9	65.7	106	96
total carbohydrates*	g	211.6	172.9	64.5	55.2	95	110.1	318.2	320.6	203.1	157.4	276.6	77	63
fiber	g	15.9	13.6	4.8	4.1	8.7	7.2	29.6	23.2	16.0	12.4	30	53	45
SFA	g	26.8	23.1	7.2	7.2	15.4	10	43	41.5	26.8	21.9	16.7	160	138
MUFA	g	27.8	27.3	6.4	7.8	15	10.6	38	48.0	27.8	25.9	34.7	80	79
PUFA	g	10.9	8.5	1.8	6.1	1.8	4.9	19.9	11.8	10.9	8.0	13.4	81	63
cholesterol	mg	238.0	217	119.9	122.3	119.9	91.0	368.1	659.3	238	182.5	300	79	72

*differences statistically significant at $p< 0.05$

Table 4. The average supply of energetic value and core nutrients in investigated group of Polish (n=13) and Greek (n=16) men

NUTRIENTS	unit	Average supply		Standard deviation (±SD)		Minimum		Maximum		Median		Standards and recommendations – weighted mean	% of standards realisation	
		Polish men	Greek men	Polish men	Greek men	Polish men	Greek men	Polish men	Greek men	Polish men	Greek men		Polish men	Greek men
energetic value	kcal	2041.8	1819.6	332.2	382.4	1376.0	1321.0	2478.0	2711.0	2005.0	1755.0	2878.4	71	63
total protein	g	79.4	74.5	18.1	17.6	49.7	46.9	129.0	113.6	79.7	71.0	77.2	103	97
animal protein	g	53.2	50.9	15.0	13.9	31.8	29.7	95.3	76.3	50.2	48.4	25.7	207	198
vegetable protein	g	26.2	23.6	6.3	6.3	17.9	14.1	37.2	37.9	24.7	22.0	51.5	51	46
total fat	g	84.5	75.1	18.4	16.6	53.7	50.5	110.8	108.4	83.6	72.5	96.9	87	78
total carbohydrates	g	243.2	221.4	49.4	60.6	162.5	128.1	334.4	360.2	231.3	210.5	395.8	61	56
fiber*	g	21.0	16.3	6.1	4.6	12.9	8.9	33.2	27.3	20.4	16.8	30	70	54
SFA	g	29.6	26.6	6.6	5.3	19.4	18.1	42.8	36.5	29.0	26.4	20.7	143	129
MUFA	g	36.2	32.6	9.1	9.6	20.6	18.5	48.8	53.6	35.5	29.8	59.7	61	55
PUFA	g	12.9	10.8	2.9	4.2	5.6	3.8	17.3	21.1	13.4	10.0	19.2	67	56
cholesterol	mg	315.6	267.1	125.6	92.0	164.7	105.7	587.3	444.8	248.8	247.4	300	105	89

*differences statistically significant at $p< 0.05$

this was 16.3 g (54% of the recommendations) for Greek men. Thus in general, the fibre content delivered in the diet for each group was very low.

Average intakes of dietary fat was 69.9 g for the Polish group but in Greeks this was 84.5 g. When compared to men, more women's diets (respectively 38.9% and 36.8% of Polish and Greek women) met recommended standard values for this nutrient, lying within the correct range of 90-110%. In men, the corresponding values were 38.5% and 43.8% for respectively Poles and Greeks that fell within the standard 70-90% range for fats.

The daily dietary content of saturated fat acids (SFA) was respectively 28 g and 24.7 g in Poles and Greeks, which exceeded the recommendations. The values for Polish women were 160% of the recommendations and 138% for Greek women, whilst for men these were respectively 143% and 129% in Poles and Greeks. In contrast to SFA, dietary MUFA were delivered in low quantities. Polish and Greek women fulfilled the MUFA recommendations, respectively at 80% and 79% cases whereas in men this was respectively 61% and 55% for Poles and Greeks. In parallel, 33.3% of the Polish women's dietary intakes met the recommendations in 90-110% and in the 70-90% ranges.

Dietary intakes of MUFA in Greek women (36.8%), mostly fulfilled the recommendations for this fat in the 50-70% range. However some women from both groups showed MUFA intakes above the recommendations by over 130%. For Polish men, the daily MUFA dietary intake varied where 38.5% satisfied the recommendations in the 50-70% range whilst a little less (30.8%) met them in 70-90% range and the same amount at 30-50%. Dietary intakes of MUFA in Greek men fulfilled the recommendations in 50% cases, within the range of 30-50%, therefore this diet delivered the least of the aforementioned fatty acids from all of the investigated groups. Furthermore, PUFAs were not delivered in sufficient quantities, with their dietary demand being met in the 50-70 % range by respectively 33.3% and 57.9% of Polish and Greek women along with respectively 46.2% and 37.5% of Polish and Greek men.

The daily diet for Poles contained 270.5 mg of cholesterol and for Greeks 239.9 mg. Diets of Polish women delivered 238 mg cholesterol, fulfilling 79% of the recommendations, whereas Greek women's diets delivered 217 mg per day, which accounted for 72% of the recommendations. Men were found to consume cholesterol in greater amounts, with the Polish diets delivering 315.6 mg per day (105% of the recommendations) and the Greek diet delivering 267.1 mg of cholesterol meeting 89.0% of the recommendations.

Significant differences were observed in the diets of Poles and Greeks for delivered calories, carbohydrate, fibre, SFA and PUFA. For Polish and Greek women, significant differences were seen in delivered calories and total carbohydrate. However only a significant difference was seen in delivered dietary fibre for Polish and Greek men.

DISSCUSION

The daily Polish diets met the nutrient demand to greater extent than the Greek ones. Significant differences were observed in dietary calories, total carbohydrate, fibre, saturated and polyunsaturated fatty acids. Both Poles and Greek diets delivered excessive animal protein and saturated fatty acids whereas too little vegetable protein, total carbohydrate were consumed according to the recommendations.

The reason for the higher calorific content in Polish meals may be related to the higher physical activity of Poles. In a study by *Sygnowska* and *Waśkiewicz* [11] on 658 men and 671 women, with variable physical activity, those who were more physically active ate higher calorific meals compared with those not being physically active at all. A similar result was indicated in the presented study.

Significant differences were observed in dietary saturated fatty acids with Poles consuming more than the Greeks. However in both groups, saturated fatty acids delivered in the diets were above recommended levels. This being likely caused by excessive intakes of animal protein. A diet rich in saturated fatty acids is not a healthy one and can lead to cardiovascular system diseases or obesity developing.

There was also more carbohydrate and fibre featuring in Polish meals. On average, 18.1 g per day of dietary fibre was delivered by Polish meals compared to 14.8 g for the Greek meals. Nevertheless, both values are too low according to recommended levels of >30 g per day.

Similar results were obtained by *Piorecka* et al. [8] for the diets of adult women from Cracow, in which total fats and cholesterol were delivered in appropriate amounts however total carbohydrates and fibre were too low. In addition, too much animal protein was consumed, whereas the vegetable protein consumption was too low. These results are mirrored in the present work in the diets of both Poles and Greeks.

The percentage calories derived from core dietary nutrients deviated from WHO/FAO recommendations which should be 10-15% from protein, 55-75% from carbohydrates and 15-30% from fats. The nutrient deviations found in Polish and Greek diets were however similar

A 2005 study [9] demonstrated a similar structure in the dietary calories delivered by the meals of healthy women, aged 30-60 and living in Athens, who had

completed a 24-hrs food questionnaires. Their diets showed 15% of calories came from protein, 43.2% from carbohydrates and 40% from fats.

Karamanos et al. [4] obtained similar results from a breakdown of delivered calories in the meals eaten in different countries where the percentage calories derived from carbohydrates was 45% and from fats 36.5%; the exception being protein which was 13.4% and was lower than in our study.

A study by *Johansson* et al. [3] stated that a too low calorific intake in diets occurs rather commonly. They also explained that there may be several reasons for a lowered calorific value; body weight, subject age, their physical attractiveness, confidence in an interviewer as well as difficulties in remembering what a meal consisted of within the last 72 hrs. Another influencing factor often found, was the willingness and ability to share information about consumed foodstuff products with an interviewer. The presented study showed that a further and important factor should be taken into account ie. the subject's level of understanding English, (in which the questionnaire was prepared), especially for the Greeks. The study subjects were chosen at random, however they were asked prior to interview about how good their English was, which in all cases proved to be very good. Nonetheless, the necessity to translate the names of local dishes and products could make it difficult for them to easily express their thoughts and, in effect, to communicate with an interviewer.

CONCLUSIONS

1. The dietary intake of Poles and native Greeks living in Athens did not meet the recommended standards of proper nourishment. In addition, they did not fulfil the recommendations defined by the pyramid of a Mediterranean diet.
2. The Poles' and Greeks' way of nourishment was typical of Western countries where diets are rich in animal fats, animal protein, saturated fatty acids and poor in carbohydrates and fibre.

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Conflict of interest

The authors declare no conflict of interest.

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A NUTRITIONAL EVALUATION OF DIETARY BEHAVIOUR IN VARIOUS PROFESSIONAL SPORTS

Karol Pilis[1], Cezary Michalski[1], Michał Zych[1], Anna Pilis[1], Jakub Jelonek[1], Agata Kaczmarzyk[3], Wiesław Pilis[1,2]*

[1] Department of Biological and Medical Sciences, Institute of Physical Education and Tourism, University of Czestochowa, Częstochowa, Poland
[2] Department of Physiotherapy, Public Higher Medical Professional School of Opole, Opole, Poland
[3] Chair and Department of Thoracic Surgery, Faculty of Medicine, Medical University of Silesia, Katowice, Poland

ABSTRACT

Background. The types of physical exertion undertaken by weightlifters and race walkers markedly differ. This difference should also be reflected in their respective diets.
Objectives. The aim of the study was to investigate and assess the diets of professional weightlifters and race walkers, along with a comparison to the diets of those students studying physical education (PE).
Materials and Methods. Subjects were respectively 12 weightlifters, 12 race walkers and 12 physical education students whose body composition and nutrition were determined by weighing the foods that were both eaten and drunk.
Results. The study groups showed body differences, which may have arisen through dietary differences. Higher calorie diets were observed for race walkers according to body mass whilst weightlifters showed no difference with the other groups. Dietary intakes of protein, fat, and carbohydrates were however inappropriate for all groups. Vitamin and mineral intakes in weightlifters and students were within tolerable limits, but the rather aggressive taking of supplements by race walkers resulted in standard/recommended consumption levels being greatly exceeded in some cases.
Conclusions. The diets of the study groups of weightlifters and race walkers need to be corrected.

Key words: *nutrition in sport, weightlifting, race walking, food supplementation*

STRESZCZENIE

Wprowadzenie. Wysiłki podejmowane przez ciężarowców oraz przez chodziarzy są diametralnie różne i dlatego sposób żywienia powinien być różny w tych grupach.
Cel. Celem pracy było zbadanie i ocena sposobu żywienia zawodników uprawiających chód sportowych oraz podnoszenie ciężarów i porównanie go ze sposobem żywienia studentów kierunku wychowania fizycznego.
Materiał i metody. W badaniach wzięło udział 12 ciężarowców, 12 chodziarzy i 12 studentów wychowania fizycznego, u których badano skład ciała i sposób odżywiania, poprzez ważenie zjadanych i wypijanych pokarmów.
Wyniki. Badane grupy różniły się somatycznie, co mogło być wynikiem różnic w racjach pokarmowych. Chodziarze spożywali posiłki o wyższej wartości energetycznej w odniesieniu do masy ciała niż studenci a ciężarowcy nie różnili się w tym zakresie od pozostałych grup, przy czym proporcje spożywanych białek, tłuszczów i węglowodanów we wszystkich grupach były niewłaściwe. Spożycie witamin i składników mineralnych u ciężarowców i studentów mieściło się w granicach tolerancji, natomiast zbyt agresywna suplementacja stosowana przez chodziarzy doprowadziła w niektórych przypadkach do przekraczania obowiązujących norm.
Wnioski. Konieczne jest wprowadzenie korekty do sposobu żywienia badanych sportowców uprawiających zarówno chód sportowy jak i podnoszenie ciężarów.

Słowa kluczowa*: żywienie w sporcie, podnoszenie ciężarów, chód sportowy, suplementacja żywieniowa*

*** Corresponding author:** Michał Zych, Department of Physical Education and Tourism, University of Czestochowa, Waszyngtona 4/8, 42-200 Częstochowa, Poland, phone: +48 34 365 59 83,
e – mail: michal.zych@ajd.czest.pl

INTRODUCTION

In sportspersons, the body's metabolic effort expended from race walking is markedly different to that of weight lifting. This should thereby be reflected in differing dietary requirements. Race walking is an activity of moderate intensity and long duration, which stimulates aerobic metabolism and renders the body susceptible to hyperthermia and dehydration [23]. On the other hand, weight lifting activates phosphorylated sources of energy in those skeletal muscles generating both extremely high tensile development and momentary strength whenever the lifted weight bar (barbell) straddles the chest; reaching a power even up to 4786 W in competitors lifting the highest weight categories [13]. Physical exertion over long periods (eg. race walking), however results in the oxidation of free fatty acids powered by glycogen metabolism in the muscles, whose pool, being less than that for fats, becomes more rapidly depleted. Thus it is recommended that such a sports' diet should be composed of 60-70% carbohydrates [5].

Supporting evidence for these premises is provided by studies showing that top long distance runners from Ethiopia consume 64.3 ± 2.6% carbohydrates when training intensively [7]. A smaller dietary role is assigned to protein and fat intake for endurance sports. It is generally recommended that race walkers consume protein, fats and carbohydrates in the ratio 1.0:0.95:5 and that the respective calorific contribution is 13% : 27% : 60%. For weight lifters the recommendations specify a dietary ratio of 1:0.99:3.9 of protein, fats and carbohydrates of calorific contents 14% : 31% : 55% respectively [4].

Even though the duration of physical effort expended during weightlifting is very short, such comparisons suggest that weight lifters consume less carbohydrate but more protein and fats than race walkers. To those engaged in power/strength sports it is often advised to consume 1.2 – 1.7 g/kg protein /24 hrs [26]. Higher protein intakes do not appear beneficial for sportspersons as *Bill* et al. [2] demonstrated that an overconsumption leads to acidosis and excessive lowering of the internal pH, that may lead to longer recoveries after training; being of significance in twice daily weightlifting workouts.

The study aims were to investigate and assess the diets of sportspersons engaged in race walking and weightlifting together with comparing them to students studying physical education.

MATERIALS AND METHODS

There were 12 subjects in each study group of race walkers, weightlifters and PE students in whose body characteristics are summarised in Table 1 and consisted of; age, height and the body's mass, fat content, water content and lean body mass along with the BMI (Body Mass Index) as measured by the Tanita Body FAT Analyzer TBF 300A, Germany in the last 5 cases. Nutritional status was assessed in all 3 groups, over 3 days, to a 2 g level of precision when determining food, supplements or water intake. Any food uneaten was subtracted from the 24 hr intake totals. Subjects were also asked about their intakes of supplements and their compositions were recorded from package information. Supplements were those recommended most often in active sports, comprising of high-energy, vitamin and mineral supplements [12, 29]. With the exception of 5 subjects, these were used by all throughout the 3-day study period.

A computer programme '*Dieta 2.0*' was used to determine dietary nutritional values per 24 hrs as developed by the National Food and Nutrition Institute [28] that incorporated a nutritional value database for selected foodstuffs and dishes [19]. These included dietary calorific values along with the proportional mass amounts and calories delivered by protein, fats and carbohydrates. Also assessed, were dietary vitamins (A, D, E, B_1, B_2, B_3, B_6, B_9, B_{12} and C), and minerals (sodium, potassium, calcium, phosphorus, magnesium, iron, zinc, copper and manganese). The one-way ANOVA test was used to evaluate statistical differences in the body data characteristics, whereas the nutritional data were analysed by the two-way repeated measures ANOVA. In both cases, the *post-hoc Tukey* test was used with a $P<0.05$ level taken as showing significance. Based on the literature, it was assumed that the analysed variables were normally distributed. ANOVAs are recognised to be resistant to a lack of normality, provided that the means are a good representation of the central tendency as verified by the skewness and kurtosis of the data's distribution [20].

RESULTS

Subjects all had similar ages but with different body characteristics as shown by significant variations in BMI so indicating different body structure/frame types. The highest BMI was found in weightlifters, whilst the lowest was in race walkers. Such body structure differences arose from different heights, body mass, body fat and water content (Table 1).

When taking dietary supplementation into account, many of the studied variables became more statistically significant as follows; protein intake expressed in g, g/kg and kcal in weightlifters, and in race walkers carbohydrate intake expressed in g, g/kg and kcal as well as dietary calorific values in kcal and kcal/kg (Table 2).

Table 1. Body characteristics of examined subject groups (mean ± SD)

Group	Age (years)	Body height (cm)	Body weight (kg)	BMI (kg/m^2)	Fat content %	Water content %	Lean bodyn mass %
Weightlifters	22.87 ± 2.67	172.03^a ± 6.11	84.27 ± 14.86	28.45^a ± 2.01	13.80^a ± 2.11	61.89^a ± 2.00	86.20^a ± 2.13
Students	22.32 ± 1.12	179.53 ±6.58	76.61 ± 10.54	23.68^b ± 1.57	16.47^b ± 2.79	59.38^b ± 2.25	83.53^b ± 2.75
Race walkers	23.42 ± 3.07	180.47^c ±5.12	69.56^c ± 4.48	21.75^c ± 1.83	9.81^c ± 1.35	65.97^c ± 1.23	90.19^c ± 1.36

a-weightlifters vs students p<0.05; b-students vs race walkers p<0.05; c- race walkers vs weightlifters p<0.05.

Table 2. Profile of dietary macronutrient contents and calorific values, with and without accounting for taking supplements (mean ± SD).

Components	Diet			Diet + supplementation			Nutritional reference values
	Weightlifters	Students	Race walkers	Weightlifters	Students	Race walkers	
Proteins [g]	133.64^a ±27.02	106.20^b ±25.41	131.25 ±22.84	171.93^{aA} ±27.30	107.40^b ±25.91	144.94^c ±25.64	
Fats [g]	124.06^a ±32.07	95.18 ±29.43	107.65 ±22.17	124.48 ±32.86	101.32 ±29.60	121.61 ±26.55	74-84^W 74-84^S 86-93^R [17]
Carbohydrates [g]	402.98 ±73.76	342.99 ±69.23	352.66 ±54.43	467.47a ±76.56	355.42^b ±0.53	448.78^C ±60.19	130$^{W\,S\,R}$[17]
Calorific values [kcal]	3327.88^a 697.33	2698.68 ±616.19	2961.51 ±461.14	3746.71^a ±699.76	2813.48^b ±617.19	3528.47^C ±507.64	3350-3800^W 3350-3800^S 3850-4200^R [17]
Proteins [g/kg]	1.58 ±0.32	1.39b ±0.33	1.89 ±0,37	2.04^{aA} ±0.32	1.40^b ±0.34	2.08 ±0.41	0.9* 1.2-1.4** <3.0*** [17]
Fats [g/kg]	1.47 ±0.38	1.24 ±0.38	1.55 ±0.37	1.48 ±0.39	1.32^b ±0.38	1.75 ±0.42	
Carbohydrates [g/kg]	4.77 ±0.88	4.48 ±0.90	5.08 ±0.88	5.55 ±0.91	4.64^b ±0.92	6.47^{cC} ±0.97	9.6-10.6^W 8,77 11.0-13.0^R[4]
Calorific values [kcal/kg]	39.49 ±8.29	35.23 ±8.36	42.57 ±7.47	44.48 ±10.41	36.75b ±8.92	50.72^C ±8.17	39.8-45.09^W ± 7.02-7.95 43.73-49.60^S ± 6.05-6.86 53.35-60.38^R ± 3.50-3.96 [17]
Proteins [kcal]	548.60^a ±111.32	435.23^b ±104.25	538.08 ±93.68	702.43^{aA} ±112.35	440.71^b ±104.48	594.32^c ±102.21	
Fats [kcal]	1129.21^a ±297.33	865.95 ±269.57	982.54 ±201,57	1137,30 ±299,91	922,81 271,22	1101,21 ±241,34	
Carbohydrates [kcal]	1655.40 ±302,70	1407.20 ±284.73	1446.87 ±221.88	1916.45^a ±312.50	1457.29^b ±289.58	1839.74^C ±246.72	

a-weightlifters vs students p<0.05; b-students vs race walkers p<0.05; c- race walkers vs weightlifters p<0.05; A-weightlifters vs weightlifters p<0.05; B- students vs. students p<0.05; C- race walkers vs. race walkers p<0.05.
Nutritional reference values: W- weightlifters (PAL – 1.75-2.0), S- students (PAL – 1.75-2.0) , R- race walkers (PAL – 2.2-2.4). *persons not training, **sportspersons, ***sportspersons endurance sports

Table 3. Dietary calories derived from protein, fat and carbohydrates before and after supplementation, and taking into account the weight (g) ratio of protein: lipid: carbohydrate

Components	Diet			Diet + supplementation		
	Weightlifters	Students	Race walkers	Weightlifters	Students	Race walkers
Proteins [%]	20.23	19.51	22.08	22.50	19.04	20.26
Fats [%]	18.78	17.48	18.46	16.34	17.97	17.00
Carbohydrates [%]	60.99	63.01	59.47	61.16	62.99	62.74
Macronutrient ratio [protein: fats: carbohydrates]	1: 0.93 : 3.01	1: 0.90: 3.23	1: 0.84 : 2.69	1: 0.73 : 2.72	1: 0.95 : 3.31	1: 0.84 : 3.10
Proteins [kcal]	16.46	16.07	18.13	18.70	15.62	16.81
Fats [kcal]	33.88	31.97	33.11	30.28	32.71	31.15
Carbohydrates [kcal]	49.66	51.96	48.76	51.02	51.67	52.04

Table 4. Mineral contents of the diet and dietary supplements (mean ± SD)

Components	Diet			Diet + supplementation			Nutritional reference values
	Weightlifters	Students	Race walkers	Weightlifters	Students	Race walkers	
Sodium [mg]	2431.38[a] ±783.55	3482.22 ±1024.38	3200.86 ±1507.09	2463.94 ±801.01	3497.93 ±1126.66	3540.68 ±1603.89	AI=1500 mg/d [17]
Potassium [mg]	3714.05 ±1245.35	3279.92[b] ±804.46	4683.69 ±1398.58	4118.63 ±1288.38	3335.85[b] ±841.56	4818.41 ±1480.56	AI=4700 mg/d [17]
Calcium [mg]	885.59 ±543.32	776.47[b] ±335.55	1146.83 ±430.17	1003.48 ±543.76	932.25[b] ±344.87	1514.32 ±557.68	RDA=1000 mg/d [17]
Phosphorus [mg]	1854.57 ±704.94	1698.95 ±412.47	2026.54 ±602.38	2067.95 ±802.84	1735.94 ±432.56	2100.12 ±634.90	RDA=700 mg/d [17]
Magnesium [mg]	398.93 ±215.76	378.54 ±162.92	437.26 ±133.66	515.72 ±233.53	397.38[b] ±155.49	682.21[C] ±186.84	RDA=400 mg/d [17]
Iron [mg]	15.75[a] ±2.67	12.25[b] ±2.77	19.16 ±5.73	22.45[aA] ±3.21	18.01[bB] ±2.02	101.21[cC] ±20.46	RDA=10 mg/d [17]
Zinc [mg]	13.02 ±4.64	12.94[b] ±2.73	18.05 ±5.25	22.94[A] ±5.21	17.92[bB] ±3.71	26.92[CC] ±7.72	RDA=11 mg/d [17]
Copper [mg]	1.44 ±0.41	1.22[b] ±0.29	1.77 ±0.51	1.76 ±0.52	1.52[b] ±0.36	2.29 ±0.78	RDA=0,9 mg/d [17]
Manganese [mg]	5.45 ±2.21	5.75 ±2.27	5.83 ±1.91	6.31 ±2.37	6.09 ±2.45	7.03 ±2.20	RDA=3,5 mg/d [17]

a-weightlifters vs students p<0.05; b-students vs race walkers p<0.05; c- race walkers vs weightlifters p<0.05; A-weightlifters vs weightlifters p<0.05; B- students vs. students p<0.05; C- race walkers vs. race walkers p<0.05.

Table 5. Vitamin contents of the diet and dietary supplements (mean ± SD)

Components	Diet			Diet + supplementation			Nutritional reference values
	Weightlifters	Students	Race walkers	Weightlifters	Students	Race walkers	
Vitamin A[µg]	900.17 ±223.45	989.93[b] ±496.78	2441.71[c] ±1008.84	1401.45[A] ±302.15	1335.24[b] ±554.37	3990.80[cC] ±1114.57	RDA=900 ug/d [17]
Vitamin D [µg]	2.69[a] ±0.77	5.59 ±3.46	3.61 ±1.13	4.46[A] ±1.23	6.12[b] ±4.57	30.21[cC] ±10.00	RDA=15 ug/d [17]
Vitamin E [mg]	11.12 ±4.37	10.02 ±4.45	13.61 ±3.81	14.89 ±4.88	14.42[bB] ±4.39	50.02[cC] ±11.18	AI=10 mg/d [17]
Vitamin B_1 [mg]	2.67 ±1.20	1.81 ±0.90	1,79 ±0,74	2.99 ±1.21	2.02[b] ±0.91	14.19[cC] ±3.24	RDA=1.3 mg/d [17]
Vitamin B_2 [mg]	2.17 ±0.68	1.86 ±0.68	2.49 ±0.67	2.49 ±0.73	2.23[b] ±0.86	5.24[cC] ±2.50	RDA=1.3 mg/d [17]
Vitamin B_3 [mg]	27.39 ±8.39	24.55 ±8.37	29.21 ±9.93	32.33 ±8.45	28.00[b] ±7.54	57.11[cC] ±18.70	RDA=16 ug/d [17]
Vitamin B_6 [mg]	2.69 ±0.88	2.28 ±0.77	2.95 ±0.92	3.12 ±1.08	2.99[b] ±0.71	20.11[cC] ±5.05	RDA=1.3 mg/d [17]
Vitamin B_{12} [µg]	4.22 ±1.36	5.12 ±3.31	5.77 ±1.93	5.88 ±1.66	6.21 ±2.98	8.05 ±3.73	RDA=2.4 mg/d [17]
Vitamin B_9 [µg]	333.86 ±87.56	314.37[b] ±94.34	438.95 ±143.66	356.66 ±99.45	319.94[b] ±82.53	678.95[cC] ±298.22	RDA=400 mg/d [17]
Vitamin C [mg]	123.67 ±74.56	88.79[b] ±53.45	222.03[c] ±100.26	187.36[a] ±72.47	98.58[b] ±61.46	746.39[cC] ±150.46	RDA=90 mg/d [17]

a-weightlifters vs students p<0.05; b-students vs race walkers p<0.05; c- race walkers vs weightlifters p<0.05; A-weightlifters vs weightlifters p<0.05; B- students vs. students p<0.05; C- race walkers vs. race walkers p<0.05.

For mineral consumption, the taking of supplements significantly increased iron and zinc intakes in all study groups as well as magnesium intakes in race walkers (Table 4). In the case of vitamins, it was however found that taking supplements increased Vitamin A and D intakes in weightlifters and race walkers, increased vitamin E in PE students and race walkers whilst increased vitamins B_1, B_2, B_3, B_6, B_9 and C in race walkers alone (Table 5).

Protein intake, expressed in g, g/kg and calorific values in kcal, and without taking any supplementation into account, was significantly lowest in students compared to weightlifters and race walkers on the first and third occasions as well for race walkers on the second occasion. In contrast, when supplementation was included then differences in protein intake between all groups on the first and third occasions became significant, whilst they were significantly lower in students on the second occasion compared to weightlifters and race walkers.

Fat consumption (g and kcal) was seen to be significantly lower in students compared to weightlifters when supplements were excluded, however in their presence, students' intake of fats (g/kg) were lower than for race walkers ($P<0.05$). Significant lower carbohydrate intakes (g and kcal) were only observed in students compared to weightlifters and race walkers when taking supplementation into account, but when expressed in g/kg, they were significantly the highest in race walkers compared to students and weightlifters. Overall, unsupplemented students consumed fewer calorific ingredients than weightlifters ($p<0.05$). When accounting for supplements, the lowest overall calorific values were seen in students compared to weightlifters and race walkers and also weightlifters consumed more calorific foodstuffs than race walkers.

Consuming calorific foodstuff ingredients by students was lower than that for race walkers, being in turn lower than for weightlifters (Table 2). In terms of consumed mass amounts of foodstuffs and supplements, then carbohydrates were the highest followed by protein and last by fats. However, the proportion (%) of calories from the diet and supplementation was highest for consumed carbohydrates, less from fats and least from protein (Table 3).

Sodium intakes, without supplements, was significantly lower in weightlifters than students, as were intakes of potassium and calcium lower in students than race walkers, irrespective of supplementation. Students taking supplements consumed significantly less magnesium than race walkers. Iron intakes were least in students and very much lower compared to weightlifters and race walkers without supplementation, however when this is taken into account, significant differences arose between all groups. Students also had lower intakes of zinc and copper than race walkers, irrespective of supplementation (Table 4).

Race walkers demonstrated the highest vitamin A intakes regardless of supplementation compared to both students and weightlifters. The latter, when not using supplements, consumed less vitamin D than students whilst those weightlifters taking this vitamin as a supplement had lower intakes than the other groups. In subjects taking supplements, a significantly higher consumption of vitamins E, B_1, B_2, B_3, B_6, and B_9 was seen in race walkers compared to students and weightlifters. In contrast, for those not taking supplements only vitamin B_9 and C intakes were higher in race walkers compared to students and weightlifters. In fact when supplements were taken, significant differences in vitamin intakes arose between all groups (Table 5). Nutrient consumption results are presented in Tables 2, 4 and 5 set against their respective reference values.

DISCUSSION

Measured differences in body characteristics between weightlifters, students and race walkers reflect the particular forms of training undertaken for these sports. Endurance training uses up body fat reserves along with an increase in body water content leading to extreme rises in lean body mass [3], without any muscular hypertrophy (ie. low BMI, body fat but high body water content in the race walkers). Changes observed in weightlifters are typical of power/strength sports consisting of excessive skeletal muscle growth, (high BMI) together with a moderate reduction of fat body mass resulting in moderate body water content and an average increase in lean body mass.

Even though weightlifters, pre or post supplementation, consume higher calorie diets than students but similar ones to race walkers, calorific intake was similar in all groups apart from significantly lower values in students compared to race walkers ($p<0.05$). This data suggests that calorific supplementation in race walkers was significant and that the long lasting, continuous and less intensity training for endurance requires a similar calorific intake as for the short but high intensity training adopted for power/strength sports. Such trends have been confirmed by *Celejowa* [4] which showed that calorific/nutritional requirements of weightlifters and race walkers to be alike, ranging 70-77 kcal/kg. *Fudge* et al. [11] demonstrated that the elite long distance runners from Ethiopia consumed in 24 hrs 3194.56 ± 329.13 kcal (56.34 ± 5.80 kcal/kg), whereas the intake for elite race walkers from another study was 4357.38 ± 286.83 kcal (61.74 ± 4.83 kcal/kg) [25].

The current study however showed lower values at 50.72 ± 8.17 kcal/kg that likely indicates a smaller

level of fitness in the race walkers. They were in fact similar to those for the weightlifters (44.48 ± 10.41kcal/kg), who after 2 hrs training, with 10-12 minutes active participation, expended around 1600 kcal.

Protein intakes, (expressed as mass and calorific values), differed between groups with supplementation being sufficiently intense to cause differences, where students showed lower levels to race walkers and weightlifters with also the latter being higher than the former ($p<0.05$). Furthermore, such intakes were in all groups raised. The Recommended Daily Allowance (RDA) for persons not training should be 0.9 g protein/kg body mass [17] but 1.2 – 1.7 g/kg for those undertaking endurance sports training [1]; this can be raised to even 3.0 g/kg [17]. RDAs for strength sports, range 1.2-1.7 g/kg body mass [26].

In professional sport, carbohydrate intake levels are important, which for endurance sports can reach up to 10 g/kg body mass per 24 hrs. *Achten* et al. [1] has suggested that increasing carbohydrate intakes from 5.4 to 8.5 g/kg body mass per 24 hrs in runner athletes improves the effects of training. Such increased capacity from increasing dietary carbohydrate intake in athletes performing strength sports has not however been documented. The presented study shows carbohydrate intakes to be lower (ie. around 4.48-5.08 g/kg and 4.64-6.47 g/kg respectively before and after taking supplements) than those recommended for all groups. The highest intakes were in race walkers and lowest for students; outcome is consistent with the literature [21].

All of the study groups had raised levels of fat intakes compared to reference values. Together with protein and carbohydrate intake, fat intake should be adequately controlled within diets to ensure that the proper proportions of these components are maintained. For physical education students, *Celejowa* [4] has proposed a ratio of 1:0.96:4.5, (protein, fats, and carbohydrates respectively), where their corresponding calories delivered should be 13%:28%:59%. The presented data from all groups however indicate that carbohydrate intakes are too low, especially for the race walkers. This nutritional error arises from consuming too much protein, particularly by weightlifters, and a small excess of fats by race walkers and students. Similarly, *Czaja* et al. [9] have observed low level of carbohydrate intake in Polish medium and long distance runners.

It should be noted that from the 1990s, professional training for competitive sports underwent diametric changes. Training with using very heavy weights (up to 70 tons) had been adopted, whereas now this has been reduced to 30 tons but of a higher intensity of activity [4]. Likewise, this has been observed in training for endurance sport. Furthermore, weightlifters now have a tall and lean body frame and thus there is an urgent need for further studies on nutrition, the human body frame and the training routine in modern sport.

The study weightlifters, students and athletes had sodium intake higher than the predicted reference value of UL=2300 mg/24h [24], which may adversely affect the body. Students had particularly high levels that may be detrimental; however this excess may be lost through sweat for those doing training [30]. Potassium intakes were highest in race walkers but lowest in students, where in the former case they exceeded the reference range (AI=4700 mg/24h) [17].

Weightlifters and students had somewhat lowered calcium intake (the reference value being RDA=1000 mg/24h) [17]. When coupled to raise sodium intake, this may adversely affect health [22]. Calcium deficiency is in fact a frequently seen defect in the diets of sportspersons [30]. In contrast, race walkers consumed one third more calcium (1514.32 ± 557.68 mg) than weightlifters, especially for those taking supplements, which fell within the reference levels recommended for sportspersons ie. 1500 – 2400 mg/24h [4].

Phosphorus intake was higher than recommended in all groups, particularly the sportspersons (Table 3), thereby making any supplementation superfluous. High phosphorus consumption is considered to decrease the absorption of iron, zinc, copper and magnesium. Doses higher than 1500 mg/24h increase blood concentrations of calcium and parathyroid hormone (PTH) [17]. In parallel with phosphorus intake, magnesium intake should be followed to ensure there isn't any excess as seen mainly in race walkers. Such intake can cause diarrhoea leading to alkalosis, hypokalaemia, dehydration, breathing difficulties and electrocardiogram changes [17].

It was found that dietary intake of iron, zinc, copper and manganese exceeded recommended reference levels in all groups, especially when accounting for supplementation. Iron is essential for synthesising protein for oxygen transport (haemoglobin and myoglobin) as well as metabolic enzymes involved in energy production [32]. Efficient oxygen transport is vital for undertaking physical effort needed for endurance activities like race walking. For such a reason it is estimated that iron intake should be increased by 70% relative to those not doing any training [33]. Despite this, it seems that such high intake, particularly iron, is not justifiable. Studies by *Hinton* and *Sinclair* [14] have demonstrated that a 6 week iron supplementation in a 3 mg dose /24hrs is enough to prevent iron deficiency and to increase endurance in sportspersons. Although recommended intake levels of these minerals are significantly exceeded, there are no health concerns of reaching the many-fold and much higher levels for them to become toxic; therapeutic doses are also very high but are likewise nowhere near being toxic [16].

The levels of vitamin A in the study were within the normal RDA range for all groups and increased whenever supplemented. Sportspersons are however recommended to consume a twofold higher than normal intake. Higher intakes of vitamin E were also observed in race walkers. When undergoing training, vitamins A and E appear to be important, because as antioxidants, they eliminate free radicals that may change how efficiently the body functions [31]. Updated RDAs in 2012 for vitamin D have increased 3-fold and currently stands at 15 μg/person/day. This value was not attained in any of the study groups except for race walkers taking supplements. Nonetheless, one has to be careful about the toxicity of this vitamin, which arises at higher doses than those described here. Vitamin D also regulates the development and homeostasis of the nervous system, skeletal muscles and maintains proper bone structure; all significant to persons undertaking competitive sports [15].

Adequate intake of the B group vitamins are important for ensuring optimal energy production along with building and regenerating muscle tissue [34]. Our findings show higher than recommended intake levels, especially upon supplementation. An excess of B_1, B_2 and B_{12} vitamins are not harmful to the body as they are easily excreted in the urine [16] and an adequate vitamin B_2 level (1.3 mg/24 hrs) improves nervous system function. A long-lasting deficiency of vitamins B_9 or B_{12}, singly or together, may cause anaemia and to lower physical efficiency [10]. The excessive vitamin B_3 intake observed in race walkers is of no concern due to the previously given reasons.

An appropriate amount of vitamin C in the body maintains the appropriate oxidative potential of the cell, delays fatigue and it is recognised that long-term aerobic training creates oxidative stress in muscles and other tissue cells [27]. The presented study shows a dramatically high level of vitamin C intake, chiefly through taking supplements, noticeably in race walkers that took 4 times more of this vitamin than weightlifters. Nevertheless, it is suggested that persons undertaking sporting endurance disciplines may consume 100-1000 mg daily [18]. Any excess is eliminated by the urine, although this may lead to kidney stones and gastro-intestinal disorders [16].

It seems that excessive vitamins supplementation, mainly B group vitamins, are not justifiable. The increased tendency to take supplements may lead to metabolic disorders and not always improves sporting physical ability; indeed in some cases it may have quite the opposite effect. To counteract this tendency, educating trainers as well as sportspersons is needed to enhance their knowledge about nutrition and in the taking of supplements [6, 9].

CONCLUSIONS

1. The studied groups had differing body structure and composition arising from their various sporting disciplines, adopted training routines and possibly types of diet.
2. Depending on the training, daily dietary calorific intake was highest for race walkers, but similar in weightlifters and students.
3. The proportions of dietary components were abnormal and did not reflect the requirement differences from the physical efforts undertaken by weightlifters, students and race walkers; both protein and fat intakes were too high whilst carbohydrate intakes were too low.
4. Raised intakes of sodium, phosphorus, iron and zinc are not a cause for concern as excess sodium is lost through sweating, the increased phosphorus (derived from highly processed food) may be easily decreased, and excessive iron arising from supplements can be limited by reducing their use whilst the high zinc intake is beneficial in conferring antioxidant properties.
5. Except for vitamin B_{12}, excessive intake of all vitamins, (due to supplementation) is superfluous and costly; even when they don't necessarily cause adverse reactions. Subject groups had deficient vitamin D intakes.
6. It is necessary to correct the diets of the studied subjects undertaking both race walking and weightlifting sports.

Conflict of interest

The authors declare no conflict of interest.

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ASSESSMENT OF NUTRITION, SUPPLEMENTATION AND BODY COMPOSITION PARAMETERS ON THE EXAMPLE OF PROFESSIONAL VOLLEYBALL PLAYERS

Joanna Zapolska, Katarzyna Witczak[*]*, Angelika Mańczuk, Lucyna Ostrowska*

Department of Dietetics and Clinical Nutrition, Faculty of Health Sciences, Medical University of Bialystok

ABSTRACT

Background. Volleyball is a team game belonging to a group of sports disciplines that involve indirect fighting. Adequate energy coverage in athletes is a crucial issue. It depends primarily on the type, intensity and duration of physical activity related to the sports discipline practiced and to the training cycle duration. The average energy requirement in sportsmen is 50% higher than that of adults.

Objective. The aim of the study was to characterize the mode of nutrition, including dietary supplements and to assess somatic indices in female volleyball players of the AZS Bialystok team.

Material and methods. The study involved 17 women. Research tools included a questionnaire consisting of 24-hour recall, a questionnaire survey concerning supplement intake and body composition analysis performed using a bioimpedance analyzer InBody 220.

Results. Data analysis indicates that the anthropometric characteristics and body composition of the AZS Bialystok players meet the recommendations associated with the somatic features in volleyball. Daily diet of the volleyball players were of low-energy with regard to the recommendations for physically active people, with very low supply of carbohydrates and dietary fiber, excessive proportion of saturated fatty acids and dietary cholesterol, and too low content of monounsaturated and polyunsaturated fatty acids. Supply of vitamins and minerals was found to be alarmingly low, especially of iron and calcium; diet supplementation was insufficient. No significant abnormalities were noted in body composition of the study athletes. However, they are recommended to increase muscle mass and slightly reduce body fat.

Conclusions. Results of diet evaluation show the need for education in the field of nutrition and the necessity of further research into dietary habits among sportsmen.

Key words*: nutrition, volleyball, dietary supplements, body composition analysis*

STRESZCZENIE

Wprowadzenie. Piłka siatkowa jest grą zespołowego współdziałania, z grupy dyscyplin sportowych o charakterze walki pośredniej. Odpowiednie pokrycie zapotrzebowania energetycznego sportowców jest bardzo istotne. Zależy ono przede wszystkim od rodzaju, intensywności i czasu trwania aktywności fizycznej, związanej z uprawianą dyscypliną sportu i okresem cyklu treningowego. Średnie zapotrzebowanie energetyczne sportowców jest o 50% większe niż osób dorosłych.

Cel. Celem niniejszej pracy była charakterystyka sposobu żywienia z uwzględnieniem suplementacji diety oraz ocena wskaźników somatycznych grupy zawodniczek klubu siatkarskiego AZS Białystok.

Materiały i metody. Badaniem objęto 17 kobiet. Narzędziami badawczymi był kwestionariusz ankiety składający się z wywiadu 24 godzinnego oraz kwestionariusz ankiety dotyczący spożywanych suplementów, a także wyniki analizy składu ciała wykonane metodą bioimpedancji elektrycznej z wykorzystaniem analizatora InBody 220.

Wyniki. Analiza uzyskanych danych wykazała, że cechy antropometryczne i skład ciała zawodniczek AZS Białystok jest zgodny z zaleceniami dotyczącymi cech somatycznych w siatkówce. Stwierdzono, że dzienne racje pokarmowe badanych zawodniczek były niskoenergetyczne w stosunku do zaleceń dla osób aktywnych fizycznie, ze zbyt niską podażą węglowodanów oraz błonnika pokarmowego, a także ze zbyt wysokim udziałem nasyconych kwasów tłuszczowych i cholesterolu pokarmowego, a zbyt niskim jednonienasyconych i wielonienasyconych kwasów tłuszczowych. Wykazano niepokojąco niską podaż witamin i składników mineralnych, w szczególności żelaza i wapnia oraz niewystarczającą suplementację diet badanych siatkarek. Nie stwierdzono istotnych nieprawidłowości w składzie ciała badanych zawodniczek. Zaleca się jednak dążenie do zwiększenia masy mięśniowej oraz nieznacznej redukcji zawartości tkanki tłuszczowej.

[*]**Corresponding author**: Katarzyna Witczak, Department of Dietetics and Clinical Nutrition, Faculty of Health Sciences, Medical University of Bialystok, ul. Mieszka I-go 4B, 15-054 Białystok, Poland, phone/fax: +48 85 732 82 44, e-mails: witczak-katarzyna@o2.pl or zdiet@umb.edu.pl

Wnioski. Wyniki oceny jadłospisów wskazują na potrzebę edukacji żywieniowej badanych kobiet oraz konieczność dalszych badań nad oceną żywienia sportowców.

Słowa kluczowe: *żywienie, siatkówka, suplementy diety, analiza składu ciała*

INTRODUCTION

Volleyball is a team game belonging to a group of sports disciplines that involve indirect fighting. The time of each match is indefinite; there is a specific scoring system, limited contact with the ball and rotations in positions. It is an interval game, classified as a technical game, and in terms of motoric features belongs to a group of strength and speed sports [31].

Adequate energy coverage in athletes is a crucial issue. It depends primarily on the type, intensity and duration of physical activity related to the sports discipline practiced and to the training cycle duration. The average energy requirement in sportsmen is 50% higher than that of adults.

The vast majority of sports disciplines (including team sports) are characterized by submaximum and maximum intensity of physical efforts, both of training and startup. In this case, the source of energy is almost always carbohydrates, while the ultimate nature of glycogen transformation (aerobic and anaerobic) is determined by the intensity and duration of physical activity, and the degree of fitness of the player [3].

The aim of the study was to assess nutrition, supplementation and body composition parameters in professional female volleyball players.

MATERIALS AND METHODS

The study involved 17 professional volleyball players aged 16-36 years. Training schedule set by the trainer and adjusted to the period of training and startup was applied. Table 1 shows training frequency of the study group. All the players in the team practiced at the same times. Training I (main) took place in the sports hall, whereas training II in a fitness club. The average training time during the entire week was 15.5 hours.

Table 1. The frequency of trainings in the study group

	Training I	Training I duration	Training II	Training II duration
Monday		2.5h	-	
Tuesday		1.5h		2.5 h
Wednesday		2.5 h	-	
Thursday		1.5 h		2.5 h
Friday		2.5 h	-	
Saturday	-		-	
Sunday	-		-	

The study involved assessment of nutrition and dietary supplements, as well as body composition. The first part of the questionnaire was based on the evaluation of dietary habits and the composition of daily food intake [29]. In the second part, data referred to dietary supplements using the method of the respondents. Body composition analysis was performed using In Body 220 (Firma Biospace). Bioelectrical impedance is a noninvasive method of measuring body composition

The analyzer determines the following parameters of body composition: TBW – Total Body Water (kg), Protein Mass (kg), Mineral Mass (kg), Fat Free Mass (kg), Body Fat Mass (kg), Body Mass (kg), Skeletal Muscle Mass (kg), BMI – Body Mass Index, Percentage of Body Fat, Waist- hip ratio (WHR), Weight Control (kg), Fat Control (kg), Muscle Control (kg), Fitness Score and Basal Metabolic Rate (kcal).

The basis of bioelectrical impedance analysis (BIA) is the use of knowledge about the human body. It consists of conductors and nonconductors. 50-70% of the human body is water, which is a conductor and fat tissue – nonconductors. The basic principle of body composition analyzer InBody220 is to determine the water content in the body and electrolytes using impedance. The water content allows determining free fat mass. Fat mass is calculated by subtracting FFM from the total body weight [8].

The questionnaire survey and analysis of dietary supplementation were performed once at the beginning of the preparatory season. Questionnaires were created in the Department of Dietetics and Clinical Nutrition on Medical University in Białystok. Nutrition standards introduced by *Jarosz* [15] were applied. The analysis of body composition performed with bioelectrical impedance was conducted three times: at the beginning of the preparatory season, in the early season and at the end of the first round of matches.

Microsoft Excel spreadsheet by Microsoft(R) Office 2003 was used for data collection. Statistical analyzes were performed using Statistica 7.1 PL. The basic descriptive statistics was performed, including means (M), standard deviations (SD), and level of significance of the difference between the respective parameters determined by body composition analysis. The probability values of $p < 0.05$ were considered statistically significant.

RESULTS

Training frequency and duration of each session can affect the variability of body composition. Anthropometric indices of the women are shown in Table 2. The mean age in the study group was 24.8 years; the average height was 180 cm. The mean body weight in subsequent studies was: 74.7, 74.4 and 73.9 kg, respectively. The average BMI values across the group in subsequent studies were 22 kg/m^2, 21.9 kg/m^2, 21.8 kg/m^2, respectively. The average WHR indicator in subsequent measurements was: 0.82, 0.83 and 0.84.

Table 2. Anthropometric indices of the study group

Parameters	No. of valid	Mean ± SD	Range
Age	17	24.8 ±5.00	16.0 – 36.0
Height (cm)	17	180.0 ± 10.00	168.0 – 196.0
Body weight 1 (kg)	17	74.7 ± 9.00	59.6 – 94.4
Body weight 2 (kg)	17	74.4 ± 8.70	57.4 – 91.0
Body weight 3 (kg)	17	73.9 ± 8.80	55.7 – 89.7
BMI 1 (kg/m2)	17	22.0 ± 1.70	19.5 – 25.6
BMI 2 (kg/m2)	17	21.9 ± 1.70	19.3 – 24.9
BMI 3 (kg/m2)	17	21.8 ± 1.70	19.0 – 24.6
WHR 1	17	0.8 ± 0.04	0.7 – 0.9
WHR 2	17	0.8 ± 0.06	0.7 – 0.9
WHR 3	17	0.8 ± 0.05	0.7 – 0.9

In the prestart period, there was no BMI < 18.5 kg/m^2. The BMI value of 18.5–24.9 kg/m^2 was noted in 15 women (88%), whereas above 25 kg/m^2 in 2 players (12%).

In the second and third studies, reductions were noted in BMI compared to the first one, the differences being 0.11 kg/m^2 and 0.24 kg/m^2, respectively; however, they were not statistically significant. BMI in the second and third test was found to be within the normal range (18.5-24.9 kg/m^2) in all the women (n = 17).

Results of body composition analysis from three consecutive tests are shown in Table 3.

During subsequent measurements, weight loss was noted in all the women (the difference was not statistically significant). Between the first and second measurement the mean weight loss was 0.3 kg, between the second and third measurement 0.5 kg, and between first and third measurement 0.8 kg.

Changes in the percentage of body fat (PBF) were also checked in the three training periods. PBF below the recommended level (<17%) was observed in 7 women in the first and third study, and in 6 in the second one. PBF within the recommended standards (17-22%) was noted in 6 volleyball players in the first and third test, whereas in 7 in the second one. The percentage of body fat was higher than the recommended level (>22%) in 4 players in all the studies.

Statistically significant changes were found in body fat mass obtained in the three measurements in different periods of training in AZS Bialystok players. In the second study there was a reduction in body fat mass by 0.6 kg, and in the third by 0.4 kg. Between the first

Table 4. The average intake of energy and nutrients in daily food intake

	Mean N=17	Range	SD	% realization of the standard and recommendations
Energy (kcal)	1909.6	(841-2729.7)	560.1	59.6
Protein (g)	113.5	(50.6-157.9)	28	94.6
Carbohydrates (g)	221.5	(76.3-366.7)	101	46.1
Fat (g)	69.9	(19.8–98)	25.9	78.6
Fiber (g)	19.8	(12–31.8)	5.8	79.2
Water (ml)	2052.8	(1041–3462)	800.4	-
Fe (mg)	12.1	(3.47–20.9)	8.65	67
Ca (mg)	728	(373–1354)	290	72.8
K (mg)	3362.2	(1747–5641)	1611	71.5
Na (mg)	3756.2	(1535–7615)	1823	250
Mg (mg)	320	(140–501)	180	103
Vitamin C (mg)	64	(128–13.9)	50	71
Vitamin B_{12} (µg)	5.92	(3.5–8.3)	2.42	246
Folic acid (µg)	239.2	(110.6–569)	128.6	59.8
Riboflavin (µg)	2.38	(0.17–6.2)	2.1	2.16
Vitamin D (µg)	6.39	(2–11.2)	4.39	127.8
Vitamin E (mg)	8.78	(3.48–16.2)	5.3	109.7
% energy from protein	21.3	(16-33.6)	6.3	-
% energy from carbohydrates	45.9	(919.8-68.8)	15	-
% energy from fat	32.8	(17.7-51.5)	10	-

Table 3. Analysis of body composition in 3 studies

Parameters	Study 1 Mean (range) N=17	Study 2 Mean (range) N=17	Study 3 Mean (range) N=17
Body weight (kg)	74.7 (59.6 – 94.4)	74.4 (57.4 – 91.0)	73.9 (55.7–89.7)
Protein (kg)	11.9 (10.0 –13.6)	12.1 (10.0 – 13.4)	12.0 (10.0–13.6)
Minerals (kg)	4.4 (3.5 – 5.1)	4.4 (3.4 – 5.0)	4.32 (3.5–5.1)
Fat mass (kg)	14.3 (7.0 – 25.9)	13.7 (6.9 – 19.8)	13.3 (7.0–23.4)
Muscle mass (kg)	34.0 (28.2 – 38.0)	38.1 (29.3 – 39.3)	36.9 (27.2–38.5)
Adipose tissue (%)	18.5 (11.2 – 29.2)	18.1 (11.4 – 25.3)	17.7 (12.0–26.1)
PPM (kcal)	1670.7 (1476.0–1850.0)	1691.0 (1462.0 –1829.0)	1735.5 (1486.0–1850.0)

*statistically significant differences

and third study, a 1 kg decrease was noted on average in body fat mass.

A quantitative dietary assessment was also performed. The energy rate of daily food intake was 1909.6 ± 560 kcal on average, accounting for 59.6% of the standard daily energy intake, whereas calorie deficiency fluctuated around 1290 kcal with regard to the recommended norms (3200 kcal). The average intake of energy and nutrients in daily food intake is shown in Table 4.

We found that the mean dietary intake of total protein was 113.5 ± 28 g, mean fat content 69.9 ± 25.9 g, carbohydrates 221.5 ± 101 g, fiber 19.8 ± 5.8 g and water 2052.8 ± 800 ml. The minimum water supply was 1041 ml, whereas the maximum 3462 ml.

The mean dietary intake of fatty acids is shown in Table 5.

Table 5. The mean intake of fatty acids and cholesterol.

	Mean N = 17	Range (min.– max.)	SD
Total fat (g)	69.9	(19.8–98,0)	25.9
Saturated fatty acids (g)	36.6	(3.9–50.71)	14.1
Monounsaturated fatty acids (g)	22.2	(2.7–48.3)	19.9
Polyunsaturated fatty acids (g)	6.0	(1.9–16.3)	4.8
Cholesterol (mg)	390.6	(125.8–773.2)	243.2

Accurate distribution of meals throughout the day is essential for proper functioning of the body. In the study group, one person had only two meals a day, six athletes had 3 meals, six women consumed 4 meals, and five had 5 meals per day. A total of 94% of the study group consumed at least three meals a day. Breakfast consumption was declared by 100% of the respondents.

Dietary supplementation was also analyzed in the study group. The use of isotonic drinks was declared by 13 athletes and regular use of supplements designed for sportspersons was declared by 12; irregular intake was reported by three women, while two respondents denied supplementation.

The assessment also referred to the most frequently consumed type of supplements designed for athletes. It was found that 71% of the study women regularly used protein rich supplements (> 80% protein), 24% used carbohydrates i.e. the so called "carbs". The use of protein-carbohydrate nutrients, as well as carbohydrate--protein supplements of "gainer" type was noted in 6% of the respondents. Consumption of vitamin and mineral supplements was declared by 71% of the volleyball players. All the athletes admitted their intake only in autumn and winter. The supplements that were consumed to improve efficiency and strength included branched chain amino acids (BCAA; consumed by 12% of the women surveyed), sets of amino acids (76%), creatine supplementation (12%) and glutamine (1 person; 6%).

The consumption of energizing supplements was declared by 47% of the respondents. The most commonly used stimulants in the study group were: caffeine (65%), caffeine tablets (41%); more rarely energy bars (24%) and guarana (8%).

The use of slimming supplements was declared by 47% of the women surveyed. The most frequently consumed supplement was L-carnitine (35% of the respondents); the consumption of chromium, CLA and green tea extract was reported by 18% and HCA by 6% of the respondents.

DISCUSSION

For a long time, coaches and trainers have been looking for factors that determine high sports achievements in volleyball. As a result of many scientific observations, a fitness model of high quality volleyball athletes has been formulated. The somatic determinants for a volleyball champion include: above-average body height, slim figure and a considerable length of limbs [16].

A substantial impact of physiological factors on the general level of physical preparation of young volleyball players has been shown by *Stamm* et al. [26], who analyzed their influence on sports results using seven objective tests. The results of all the tests showed a close correlation with anthropometric characteristics.

Body composition as a predisposing factor has been considered in Melrose study. The ideal physiological profile of a volleyball player, which can facilitate the assessment of normal weight and somatic type, is an important factor in achieving high performance in sports [4, 24]. In addition, information on body composition indicates nutritional habits of the player and provides information about body homeostasis [3]. The knowledge of body weight and its individual components can be used as guidance for trainers to make adjustments in the diets of athletes [13].

Body composition of highly skilled athletes should differ significantly from that of the general population, even when compared with people who practice sports recreationally. The highest quality athletes have an increased percentage of lean muscle mass and lower percentage of inactive mass (fat) [21].

During tournaments, players of two female volleyball teams often compete for the ball over the net, which is suspended 2.24 meters above the ground. In the current study, the mean body height was 180 ± 10 cm, being higher than the average body height of the first league female volleyball players from Greece - 176.1 cm [13], Japan - 168.7 cm [32] and India - 159.67 cm [17]. A similar average body height was noted in Spanish volleyball players - 179.7 cm [12], American - 177.9

cm [5], and Brazilian ones - 174.4 cm [1]. Higher body height was reported in the Polish athletes from "Gedania" club in Gdańsk - 182.7 cm [27], and in players from Czech Republic - 184 cm [22].

Currently, the most common method used to assess nutritional status is by means of Body Mass Index (BMI), which is easily available and simple. However, BMI takes into account only the total body weight, without identifying its components, such as muscle and bone tissue, fat tissue and body fluids. This method does not distinguish between the somatic types. In the present study, according to BMI, the players were not overweight and showed no risk of obesity (only in two athletes slight excess was reported), 88% of the study players was well nourished. Some sources show a strong correlation between BMI and body fat [4].

In the current study, the average content of body fat was 18.5%. The result is satisfactory, since the values obtained are similar to those of high performance athletes from other countries. According to literature data, the national elite athletes from Czech Republic showed the lowest percentage of body fat (15.9%) [22]. However, a similar PBF compared to the current results was observed in the university volleyball team surveyed by *Tsunawake* et al. (18.4%) [32]. A much higher percentage of body fat was found in the volleyball team examined by *Abreu de Almeida* et al. (20.5%) [1], *Melrose* et al. (21%) [24], *Malosauris* et al. (22.7%) [21], *Kreger* et al. (22.92%) [18] and *Frasson* et al. (24.93%) [11].

In our study, the average content of muscle mass was 34.3 kg, 38.1 kg and 37 kg in consecutive measurements, accounting for 45.5%, 51.2%, 50% of the total body weight, respectively. Similar values of body muscle mass were observed in volleyball athletes from the United States - 33 kg [30]. However, much higher values were reported from Greece - 46.7 kg [13] and Czech Republic - 55.8 kg [22].

In the present study, no significant changes were found in the BMI from the three training periods. Worthy of note is that at the beginning of the season BMI indicating overweight was noted in two volleyball players. However, in the third study, all the athletes met weight standards. Changes in body mass were not statistically significant (mean 0.8 kg).

Changes in adipose tissue turned out to be satisfactory. The average decrease in body fat was 1.0 kg (approximately 7% from the beginning of the season). The most substantial decrease recorded was 2.5 kg. Similar results were obtained by *Gonzales-Rave* et al. [12]. We found that the decrease in fat mass between the first and third test was accompanied by an increase in muscle mass by 2.7 kg on average (i.e. about 4.5% of total body weight). There is no doubt that the quality and quantity of food and drinks consumed by athletes have a significant impact on their athletic performance.

Studies conducted among young female athletes have shown that the average consumption of energy, vitamins and minerals is too low in relation to the increased needs of the body [14, 20, 25, 28, 30].

In our study, dietary habits were analyzed on the basis of three-day dietary interview conducted before the training started. The energy value and the content of the daily food intake of protein, fats and carbohydrates, as well as some minerals and vitamins, fiber, cholesterol and fatty acids were estimated. Our findings indicate that female volleyball players do not fully implement the recommendations for rational nutrition. The energy provided by daily diet of the surveyed athletes did not meet daily requirements (mean 1909.6 ± 560.1 kcal), covering the demand in 63.6%. Lower energy intake in a daily diet was reported among professional volleyball players from Howard University [30], among leading players of the India volleyball team [14], where the average energy intake was 1471 ± 479 kcal, and among volleyball athletes from Greece - 2013 ± 971 kcal [25]. Insufficient amount of energy in daily diet was also observed in the Greek Super League volleyball team - 2049 ± 735.12 kcal [2]. Not much higher daily energy intake was observed in the elite volleyball players from the USA, with an average of 2248 ± 414 kcal per day; however, the reported value was much lower than that recommended for the group [6].

Energy deficit may have a number of adverse effects. Regular shortage of energy in the diet of an athlete can lead to weight loss, which may involve the risk of muscle mass loss as an undesirable effect of training, and can degrade performance. According to the American Dietetic Association and Medicine in Sports Sciences [6], these ratios should be as high as 12-15% of energy from protein, 25-30% from fats and 55-58% from carbohydrates.

We showed abnormal energy structure of food ration, with too high proportion of fat-derived energy - 32.8% and a very low share of energy from carbohydrates - 45.9%. The share of protein-derived energy was 21.3%, being relatively high. Improperly balanced diet in terms of the content of essential nutrients in the implementation of energy demand was also observed in athletes practicing volleyball in Greece [25], India [14], as well as those in the Polish national team [27].

In volleyball, like in other sports, supply of dietary protein should be considered an important nutritional factor. Protein is an important dietary component among sports professionals - it not only determines body's adaptation to exercise and speed recovery after workout, but is also responsible for proper pace of body's development and its resistance [28]. Energy percentage of daily food ration derived from protein should be about15%, i.e. an average of 1.2g-1.4 g / kg. mc./ day.

In our study, the average overall protein intake was 113.5 ± 28 g, which covers the demand in 75.7%. Lower intakes were recorded in the Greek volleyball league menus [25], in the 2nd league athletes [30] and in the elite volleyball players in the USA [6]. There was no case where standards for this component were implemented.

Since carbohydrates are the primary energy substrate for working muscles, the recommended share of carbohydrates in daily diet is as high as 60-68% of energy. In the present study, we found much lower share of energy derived from carbohydrates (45.9%), which yielded an average of 221.5 ± 101 grams of carbohydrates a day. Similarly, low scores were noted in female footballers in Greece [25], India [14] and the USA [6].

Such amount of carbohydrates is insufficient for adequate glycogen re-synthesis in female athletes whose stocks are quickly depleted during intense training and competitions. Consequently, the player's efficiency in training or match can be significantly reduced, and may result in an earlier onset of fatigue [28].

In the current study, the amount of dietary fiber was insufficient (mean 19.8 ± 5.8 g). As specified by the World Health Organization, dietary fiber intake should be in the range 27-40g/day. Low fiber intake has been confirmed by many studies carried out in Poland, Greece and the USA [6, 25, 28]. The intake of sucrose was found to be too high (mean 69.5g), accounting for 31% of total carbohydrates.

Fat intake should be at the adequate level in order to ensure delivery of proper amounts of essential unsaturated fatty acids and fat-soluble vitamins, as well as to maintain healthy body weight. We showed that the percentage of fat-derived energy in a daily diet exceeded the norm (30%) and was 32.8%. However, the average dietary fat content was 69.9 g and implemented the standard for this nutrient in 70%. A higher intake of fat was observed in studies concerning Greek [25] and India [14] female volleyball players.

In addition, in accordance with the standards of the World Health Organization the intake of the respective fatty acids should cover the following percentages of diet energy: saturated fats 7%, monounsaturated fats 10-15% and polyunsaturated fats 6-10% [15]. In the current study, the consumption of fatty acids in the athletes included 17.23% of saturated, 10.4% of monounsaturated and 3% of polyunsaturated fatty acids.

According to the standards of the Institute of Food and Nutrition, the daily intake of cholesterol is 300 mg [15]. In our study, the mean cholesterol intake was 390.57 mg. Alarmingly, the intake of dietary fiber, which effectively reduces the level of cholesterol in the blood, was below the standard (19.8 g).

In the diets for athletes, calcium and iron intake is of special importance, as their shortage is very common and may entail adverse health consequences [2, 28]. Their daily norm coverage was calculated based on the standards established by the Institute of Food and Nutrition [15] for the recommended intake of minerals for physically active individuals. The mean iron content in daily diet was 12.1 ± 8.65 mg, which accounts for only about 40% of the minimum implementation (importantly, the absorption of this element is relatively low). The mean dietary calcium content was 728 ± 290 mg (33% of standard implementation). The content of vitamins and other micronutrients in the daily diet was below the recommended intake. Such low content of microelements was observed in daily diet of the elite female volleyball players in India [2, 14]. A higher dietary content of vitamins and minerals was observed among Polish athletes residing in Central Sports Centre [2], as well as in Greek female volleyball players [25].

The mean sodium intake in the diets was found to be 3756.2 mg and the maximum amount of sodium was 7615 mg, accounting for 508% of the norm for the ingredient.

In our study, the average fluid intake was 2052.8 ± 800.4 ml. This value is only slightly lower than is recommended (covers 80% of standard implementation); however, it should be remembered that the athlete's body has to make up for fluid loss during exercise to avoid a drop in performance and concentration. Dehydration increases body's fatigue, which results from increased glycogenolysis.

When meals are regularly distributed throughout the day, nutrients can be used properly. The current study took into consideration the number of consumed meals. The standard specifies that a well-planned menu should include 4-5 meals per day, which in the analyzed diets was admitted by about 60% of the players. Similar results were obtained in a survey of female students of Academy of Physical Activity (AWF) in Olsztyn and Warsaw in 2002 (62%) [33]. Significantly lower values were obtained in a study concerning Polish professional volleyball athletes, where the consumption of 4-5 meals was declared by only 17.4% of the respondents [10] and in a study from Podkarpackie Province concerning athletics, where 4-5 meals were consumed by 44% of women [9] .

Isotonic drinks are designed to adjust the levels of water and electrolytes excreted from the body in the process of perspiration, and to supplement vitamins, minerals and small amounts of carbohydrates to allow re-synthesis of glycogen, which decreases during exercise. The assessment of eating behaviors showed that 76% of the volleyball players supplemented fluids with isotonic drinks. This result is satisfactory, taking into account the fact that the diet of the female athletes studied did not include enough fluids.

Food components contained in nutrients and supplements positively influence the process of muscle protein recovery, as well as energy processes. Supplementing the diet with nutrient concentrates can enhance the anabolic processes of regeneration, provide enough energy for exercises and protect muscle proteins from degradation.

The surveyed athletes supplemented their diets with different formulations. The use of sports supplements was declared by 89% of the respondents. The most popular nutrients included high protein supplement (71%) and nutrient carbohydrate (24%). Vitamin and mineral supplements were shown to be used by 82% of the players daily or periodically. Lower intake of such supplements was noted in volleyball players from Krakow [10].

Creatine, which is the most studied supplement, was used by our two volleyball players. The role of creatine is to increase the concentration of phosphocreatine in the muscle cells, leading to the acceleration of ATP re--synthesis rate. This process delays the onset of fatigue and facilitates restoration process during repetition of series of high-intensity exercises [23]. Administration of creatine also has a beneficial effect on lean body mass gain, increasing both absolute and relative anaerobic strength [19].

In our study, the most frequently consumed supplements included preparations containing the entire set of amino acids (76%) and stimulants (47%), including coffee (65%) and caffeine tablets (41%). The players also consumed BCAA branched amino acids, creatine, glutamine and slimming preparations (L-carnitine, chromium, CLA, green tea).

CONCLUSIONS

1. Daily diet of the volleyball players were of low--energy with regard to the recommendations for physically active people, with very low supply of carbohydrates and dietary fiber, excessive proportion of saturated fatty acids and dietary cholesterol, and too low content of monounsaturated and polyunsaturated fatty acids.
2. Supply of vitamins and minerals was found to be alarmingly low, especially of iron and calcium; diet supplementation was insufficient.
3. No significant abnormalities were noted in body composition of the study athletes. However, they are recommended to increase muscle mass and slightly reduce body fat.

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A SUBJECTIVE DISSATISFACTION WITH BODY WEIGHT IN YOUNG WOMEN: DO EATING BEHAVIOURS PLAY A ROLE?

Katarzyna Eufemia Przybyłowicz[1], Dorota Jesiołowska[1], Małgorzata Obara-Gołębiowska[2], Lidia Antoniak[1]*

[1] Chair of Human Nutrition, Faculty of Food Sciences, University of Varmia and Masuria, Olsztyn, Poland
[2] Chair of Developmental Psychology and Education, Faculty of Social Sciences, University of Varmia and Mazuria, Olsztyn, Poland

ABSTRACT

Background. Food less frequently used to provide the necessary nutrients for the survival and the body begins to play a role, which it is not able to cope with, leading to a dysfunctional its use. In an era of obesity, excessive interest in his appearance and lean silhouette assigning too much significance relates to a growing number of young women. Young women due to a period of their procreative years are particularly vulnerable to the consequences of abnormal eating habits that threaten the health of women and their offspring.

Objectives. In young women of reproductive age, to determine the emotional and habitual reasons behind binge eating and the effect that restriction diets can have for achieving desired body mass in relation to physical activity and the willingness to improve their weight.

Material and methods. Subjects surveyed were 372 women aged 18 to 27 years (mean 20.6 ± 1.4) who answered a questionnaire on dietary behaviour devised by *Ogińska-Bulik* and *Putyński* [21] which had been extended to include body mass perception/image, adoption of slimming diets, levels of physical activity and place of residence. The women's actual body mass, height and body fat (adipose tissue content) were also measured.

Results. Most subjects (63.9%) were dissatisfied with their figures whilst 33.5% underwent slimming diets at least once. Those overweight, complained much more about their figures compared to normal weight women (97.9% vs. 65.1%, $p<0.01$), as well as being respectively more emotionally prone to overeating (4.5 ± 2.2 vs. 5.2 ± 2 points round, $p <0.01$), but less for adopting any dietary restriction (3.5 ± 2.7 vs. 4.8 ± 2.3, $p <0.01$).

Conclusions. It seems necessary to create a prevention and educational programs on proper nutrition and the perception of one's own body as effective tools in reducing eating disorders in terms of the health of young women and multigenerational inheritance health of their offspring.

Key words: *restriction diets, obesity, overweight, emotional overeating, reproductive age, pregorexia*

STRESZCZENIE

Wprowadzenie. Żywność coraz rzadziej służy do dostarczania potrzebnych do przeżycia składników odżywczych ciału a zaczyna odgrywać role, którym nie jest w stanie sprostać, co prowadzi do jej dysfunkcjonalnego zastosowania. W erze otyłości nadmierne zainteresowanie swoim wyglądem oraz przypisywanie szczupłej sylwetce zbyt dużego znaczenia dotyczy coraz większej grupy młodych kobiet. Młode kobiety ze względu na okres okołokoncepcyjny są szczególnie narażone na konsekwencje nieprawidłowych nawyków żywieniowych, które zagrażają zdrowiu kobiet i ich potomstwu.

Cel badań. Badanie i ocena emocjonalnego i nawykowego objadania się oraz stosowania restrykcji dietetycznych w relacji do subiektywnej oceny posiadanej i oczekiwanej masy ciała, aktywności fizycznej i chęci jej zmiany wśród młodych kobiet w wieku prokreacyjnym.

Materiał i metody. Badaniem objęto 372 kobiety w wieku od 18 do 27 (20,6 ± 1,4) lat. W badaniach stosowano Kwestionariusz Zachowań Związanych z Jedzeniem *Ogińskiej-Bulik* i *Putyńskiego* [21], poszerzony o ankietę własną zawierającą pytania dotyczące stosunku do masy ciała, częstości stosowania diet odchudzających, stopnia aktywności fizycznej oraz miejsca zamieszkania. Następnie dokonano pomiarów masy i wysokości ciała oraz zawartości tłuszczu w organizmie.

Wyniki. Wśród badanych kobiet 63,9% było niezadowolonych z sylwetki, a 33,5% badanych odchudzało się chociaż 1 raz. Kobiety o nadmiernej masie ciała w porównaniu do kobiet z prawidłową masą ciała były najbardziej niezadowolone

***Corresponding author:** Małgorzata Obara-Gołębiowska, Chair of Developmental Psychology, Faculty of Social Sciences, University of Varmia and Masuria, Prawocheńskiego Street 13, 10-447 Olsztyn, Poland, phone +48 89 5246229, fax +48 89 5235057, e-mail: malgorzata.obara@gmail.com

z sylwetki (97,9% vs. 65,1%, p<0,01) i wykazywały największe tendencje do emocjonalnego przejadania (4,5±2,2 pkt vs. 5,2±2 pkt, p<0,01) oraz restrykcji dietetycznych (3,5±2,7 vs. 4,8±2,3, p<0,01).
Wnioski. Konieczne wydaje się stworzenie programów prewencyjno-edukacyjnych w zakresie prawidłowego żywienia i postrzegania własnego ciała jako skutecznych narzędzi w ograniczeniu zaburzeń odżywiania w aspekcie zdrowia młodych kobiet i wielopokoleniowego dziedziczenia zdrowia u ich potomstwa.

Słowa kluczowe: *restrykcje dietetyczne, otyłość, nadwaga, emocjonalne przejadanie, wiek prokreacyjny, pregoreksja*

INTRODUCTION

Food less frequently used to provide the necessary nutrients for the survival and the body begins to play a role, which it is not able to cope with, leading to a dysfunctional its use. In an era of obesity, excessive interest in his appearance and lean silhouette assigning too much significance relates to a growing number of young women [25, 26]. Young women due to a period of their procreative years are particularly vulnerable to the consequences of abnormal eating habits that threaten the health of women and their offspring [4,14].

Being overweight adversely affects human health and so promoting a healthy nutritional lifestyle and physical activity becomes necessary. Having a slim figure is however mistakenly treated as a marker unrelated to health, serving to rather reflect personal worth. Without accounting for a person's natural predispositions, expectations of society impose a falling into line with cultural role models. From the earliest of years, girls' attentions are directed towards being slim and slender [19, 33]. This explains why obesity is regarded as something that breaks the standard views on beauty and health. As well as problems of overweight and obesity in young women, there are equally also other health threats from eating disorders arising from inappropriate and obsessive focus on personal appearance along with an uncritical yearning to conform to modern images of being attractive ie. of being a very slim woman. Further, evidence suggests that this disparity reflects, at least in part, the fact that the prevalence of body dissatisfaction and eating-disordered behaviour is higher - and the adverse effect of these variables on mental health greater - in overweight women than in overweight men [18,19].

Women of reproductive age are not only responsible for shaping their health but that of future generations. Disorders of eating, before or during pregnancy, are known to be risk factors for the poor health of future mothers and their offspring [3]. Furthermore, slimming during pregnancy can lead to pregorexia which in turn may cause premature birth and lower newborn weight [4, 14]. Foetal malnutrition increases the likelihood of obesity and metabolic disorders in later life. Many studies focus on the effects of overweight on poor health and in finding its aetiology. Few studies however look at the mental, emotional and habitual aspects of either overeating or adopting excessive restriction diets. In Poland, there are scarcely any studies on behavioural aspects of nutrition in women of reproductive age and thus it is important that body/figure image is studied in relation to dietary behaviour.

The presented study therefore aims to investigate emotional and habitual overeating, together with adopting restriction diets in relation to body mass (existing and desired) and the willingness for this to change in young women of reproductive age; including also assessing physical activity.

MATERIAL AND METHODS

Sampling

Subjects were 372 women of 18 to 27 years age (20.6 ± 1.4) recruited from the University of Varmia and Masuria in Olsztyn and living at various urban centres; 62.7% towns and 37.3% countryside. The Rolling Snowball (referral) sampling method was used, where existing respondents recommended their fellow peers that fulfilled the study criteria. These were an absence of mental illness, chronic metabolic diseases, pregnancy and lactation as well as agreeing to participate in the study and any further follow up as and when required. Questionnaires were filled in after the study aims had been explained by suitably qualified staff.

Anthropometric measurements and assessment of body composition

These consisted of height, body mass and composition. Subjects were measured in the 'Frankfurt Plane' (standard anatomical position) without footwear and outermost clothing. Nutrition was assessed by BMI, according to WHO classification; BMI ≤ 18.5 (undernourished), BMI 18.5 ≤ BMI < 25 (normal body mass) and BMI ≥ 25 (overweight/obese). The body composition was determined by FUTREX 6100/XL measurement. The body fat content was divided into three categories; low fat (< 25%), normal fat (25 - 30%) and high fat (> 30%). The precision of all measurements was 0.1%.

Questionnaire

This consisted of the Nutritional/Eating Behaviour Questionnaire (*Kwestionariusz Zachowań Związa-*

nych z Jedzeniem; KZZJ) devised by *Ogińskia-Bulik* and *Putyński* [21] which is a universally used tool for studying the Polish population regarding dietary/ nutritional behaviour, that includes diagnosing eating disorders when dieting and predicting the likelihood of weight gain. It is composed of 30 statements with either yes or no answers. Depending on the question, each reply is assigned a mark of 0 or 1 point, where the total points per block, allowed nutritional behaviour to be graded accordingly. Eating abnormalities were defined as ranging between 0-10 points for each of the 3 blocks, respectively; emotional and habitual overeating as well as the tendency to adopt restriction diets. The higher number of points scored, then the greater is the eating behaviour abnormality. The questionnaire was extended by the study authors to include details on body mass, how often were slimming diets adopted, physical activity levels and places of residence.

Statistical analysis

Results were usually expressed as means (x) and standard deviations (SD) for each studied characteristic/ variable. Mean values were compared by the *Kruskal--Wallis* test and distributions by the *Chi* squared test ($\chi 2$). Correspondence analysis was carried out for the 8 characteristics and results presented in a two dimensional coordinates form, which explained 21% of the inertia. Results were calculated by the Statistica 10.0 PL programme and significance was taken at the $p \leq 0.05$ level.

RESULTS

Presented research constitutes a preliminary part of survey program related to behavioral conditions of young women's nutritional status.

Women's nutritional status

Subjects had normal BMI values of 21.7 ± 3.0 (min-14.9, max-34.2) and normal body fat percentage 28.0 ± 4.8 (min-15.0, max-43.5). Underweight was observed in 9.9% women, normal body mass in 75.9% and overweight in 14.2%; Table 1**.** Significant differences were seen between BMI and body weight with the frequency of adopting diets and physical activity, but none with places of residence. Of those undernourished women, 42.2% expressed satisfaction with their body mass, 84.8% were never on slimming diets and 72.7% declared an average/medium level of physical activity. In women with a normal body mass, 53.6% wanted to be a little slimmer, 59.9% did not adopt slimming diets and 57.5% engaged in average levels of physical activity. For those who were overweight/obese, 61% would like to be slimmer and 44.7% had low levels of physical activity as did a similar proportion who un-

Table 1. BMI (body mass index) and body's adipose (fatty) tissue content.

Parameter	BMI [kg/m^2]			Body fat [%]		
	>18.5	*18.5-24.9*	*>25*	*<25*	*25-30*	*>30*
Numbers [N]	45	282	53	90	190	92
Sample percentage [%]	9.9	75.9	14.2	26.5	46.7	26.8

Table 2. Body image, eating behaviour and physical activity regarding BMI

	Total [%]	Malnourish-ment [%]	Correct body mass [%]	Overweight and obesity [%]	p
Relationship to own body mass					
Desire to be a lot slimmer	17,5	0	11,5	61,7	p>0.01
Desire to be a little slimmer	46.4	6.1	53.6	36.2	
Desire to gain a little weight	7.2	33.3	5.2	0	
Desire to gain a lot of weight	0.6	6.1	0	0	
Reasonably satisfied with body mass	23.5	42.4	25	2.1	
My body mass is ideal	4.8	12.1	4.8	0	
Frequency of dieting					
1-2 times	13.6	9.1	12.7	21.3	p>0.01
3-5 times	13	0	13,1	21.3	
6-10 times	3.3	0	2.4	10.6	
>10 times	3.6	3	3.6	4.3	
Don't know	9.6	3	8.3	21.3	
Never	56.9	84.8	59.9	21.3	
Physical activity					
Low	30.7	15.2	30.2	44.7	p=0.05
Medium	56.6	72.7	57.5	40.4	
High	12.7	12.1	12.3	14.9	

Table 3. Body image, eating behaviour and physical activity regarding Eating Behaviour Questionnaire

	General indicator [x ±SD] [0-30 pts]	Habitual overeating [x ±SD] [0-10 pts]	Emotional overeating [x ±SD] [0-10 pts]	Restriction dieting [x ±SD] [0-10 pts]
Total	11.5±5.6	3.6±2.5	4.4±2.2	3.5±2.7
BMI [kg/m^2]				
<18.5	7.2±4	2.8±2.3	2.8±1.7	1.5±1.7
18.5-25	11.7±5.7	3.7±2.5	4.5±2.2	3.5±2.7
>25	13.4±4.9	3.4±2.6	5.2±2	4.8±2.3
	p<0.01	p=0.1	p<0.01	p<0.01
Relationship to own body mass				
Desire to be a lot slimmer	16.6±5.1	4.8±3.1	6.1±2.1	5.7±2.2
Desire to be a little slimmer	12.9±4.8	3.7±2.3	4.9±2	4.3±2.4
Desire to gain a little weight	8.3±4.5	4±2.5	3.2±2.1	1±1.3
Desire to gain a lot of weight	9±0	4±0	1±0	4±0
Reasonably satisfied with body mass	7.1±3.2	2.7±2.3	3±1.5	1.4±1.7
My body mass is ideal	6±3	2.4±1.9	2.3±1.6	1.3±1.2
	p<0.01	p<0.01	p<0.01	p<0.01
Frequency of dieting				
1-2 times	11.6±4.4	2.7±2.4	4.8±1.9	4.1±2.8
3-5 times	14.8±4.8	3.8±2.3	5.5±2.2	5.6±2.3
6-10 times	18.8±4.5	5.5±2.5	6.8±1.8	6.5±2.1
>10 times	18.9±6.2	5.8±3.5	5.9±2	7.3±2
Don't know	14.7±5.1	4±2.5	5.1±2.4	5.6±2.1
Never	9.3±4.7	3.4±2.4	3.7±2	2.1±1.8
	p<0.01	p<0.01	p<0.01	p<0.01
Physical activity				
Low	12.9±5.7	4.5±2.9	4.9±2.4	3.4±2.5
Medium	10.8±5.4	3.2±2.3	4.2±2.1	3.4±2.7
High	11.3±5.9	3±2.3	4.2±2.3	4.2±2.9
	p=0.01	p>0.01	p=0.03	p=0.2

dertook slimming diets; 1-2 times and 3-5 times (both 21.3%); Table 2.

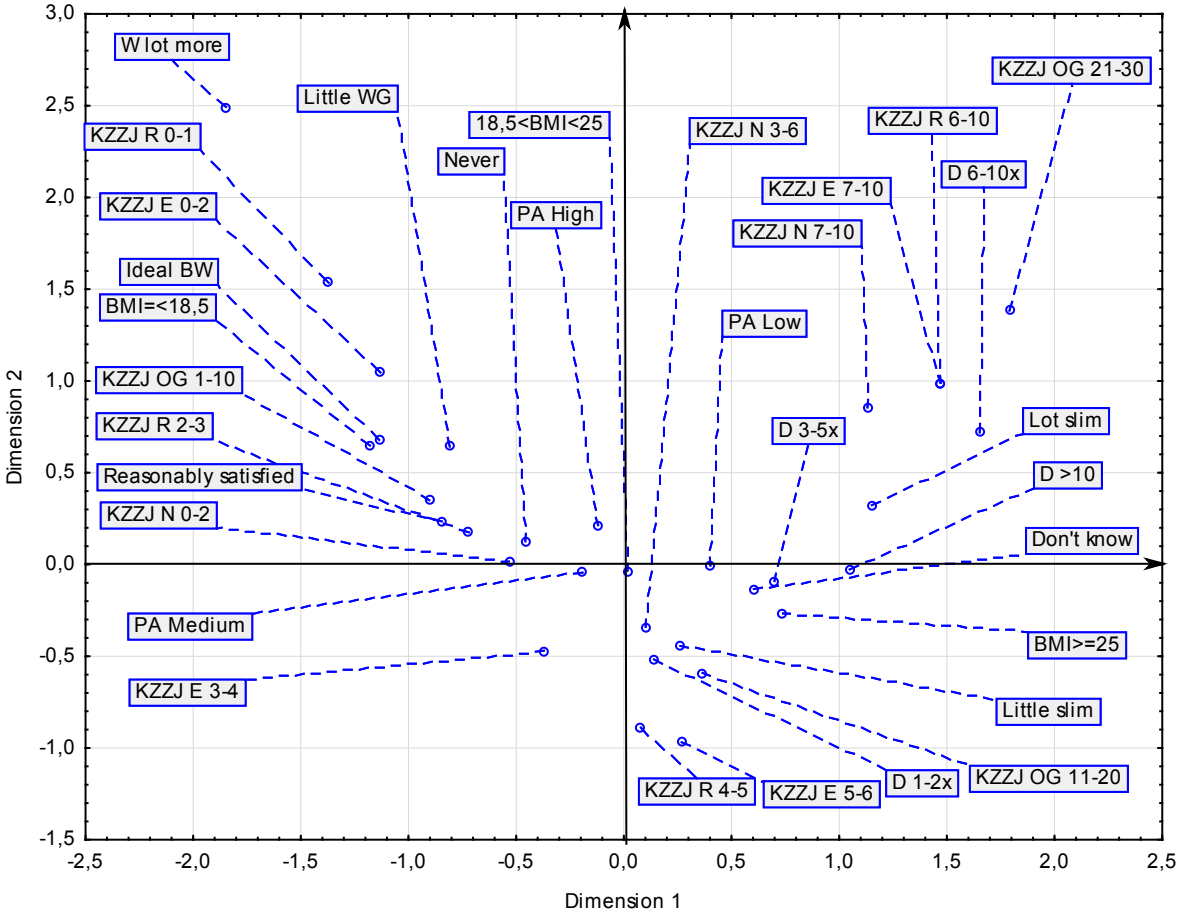
In overweight and obese women, the BMI was found to be associated with abnormal nutritional behaviour, emotional overeating and being on restricted diets. The greatest abnormalities in nutritional behaviour, emotional and habitual overeating as well as being on restrictive diets were observed in those women wishing to be a lot slimmer. Similar findings were seen in subjects who tried slimming at least 6 times or more. Low levels of physical activity were found to increase the risk of abnormal nutritional behaviour, including the emotional and habitual overeating. There were no significant differences between habitual overeating with BMI, physical activity and restricted dieting (Table 3).

The correspondence analysis found that underweight women were more frequently satisfied with their figures and more willing for gaining a little weight compared to normal weight women or those overweight or obese (Figure 1). This former group were less predisposed to abnormal nutritional behaviour such as the emotional overeating or adopting restrictive diets. Overweight and obese subjects more often tried to slim down, had lower physical activity levels and were more eager to be slimmer than the other subject groups (Figure 1). Those overweight showed greater abnormal nutritional behaviour, where they frequently overate emotionally and habitually, at the same time as being on restrictive diets.

Women with normal body mass claimed that they more rarely tried to slim than those overweight, resulting in lesser risk of abnormal nutritional behaviour in the former (Figure 1). This group was also more satisfied with their figure than the overweight and obese as well as undertaking more physical activity. Nevertheless they still wished to be more slim.

DISCUSSION

Emerging evidence suggests that body satisfaction may be such a leverage point [13]. The relationship between poor body satisfaction and increased risk of onset of disordered weight control behaviors and symptoms, including vomiting, fasting, and use of laxatives and diet pills for weight control, has been well-established in prospective studies with adolescent females and males [29].



Description: FM – Body fat mass, PA – Physical activity, D – Frequency of dieting, KZZJ OG – General results of the Nutritional/Eating Behaviour Questionnaire (KZZJ), KZZJ E – Results of emotional overeating from the KZZJ, KZZJ N - Results of habitual overeating from the KZZJ, KZZJ R - Results of restrictive dieting from the KZZJ

Figure 1. Relationships between nutritional status, body image, eating behaviour, physical activity and dietary habits according to Eating Behaviour Questionnaire

Abnormal nutritional/eating behaviour, including overeating, arises from many contributing sources such as from emotional states, habits and body image perception [18, 27]. Investigators seeking the causes and treatment of disorders in body mass are increasingly taking into account psychological factors. Interestingly, the association between body weight and mental health impairment in women does not appear to be confined to overweight. Rather, findings from those studies that have considered associations with mental health across the full spectrum of body weight suggest the presence of U-shaped relationships, such that both underweight and overweight are associated with poorer mental health in women [13]. In other studies, only underweight has been found to be associated with mental health impairment [17].

There have been many changes in Poland over the last 23 years, that include cultural ones and in the patterns of human consumption. A much greater availability and diversity of foodstuffs combined with an ubiquitous media presence have resulted in two contradictory messages; 'eat' and 'be slim' [10]. Lifestyle changes resulting from technological progress have additionally limited physical activity which had previously been the mainstay of preventing obesity. Studies by *Roberts* and *Good* [23] have demonstrated that neurotic women are more susceptible to images of slim figures in the media. An analysis by *Rybicka-Klimczyk* and *Brytek-Matera* [24] on women's satisfaction with their figures, of various age, showed that those aged 20 – 25 were most dissatisfied and most often adopted slimming diets.

Similar findings have been noted by both *Schwartz* and *Brownell* [26] and *Brytek-Matera* and *Charzyńska* [6], where obese women expressed greater dissatisfaction about their appearance and more frequently took steps for slimming their figures down compared to women with normal body mass. Likewise, a study on Brazilian female students found more dissatisfaction about their figures in those that were obese, with 73% of these downgrading their actual body mass [12]. It has been proved that reducing the body mass leads to a less distorted figure, but not in those that are obese during

adolescence. It seems that one's figure becomes fixed at this time and is difficult to then change [30].

Perceiving one's figure as being unattractive through comparison with stereotypes promoted by the media can be stressful [2]. The presented study has demonstrated that women who are overweight or obese are more likely to emotionally overeat. The findings also suggest that women who are very underweight are a vulnerable group, being at increased risk of impairment in both physical and mental health [20, 31]. One of the contributing factors is stress which causes cortisol levels to rise and so deregulates the brain's reward centre and leads to the overconsumption of highly palatable foodstuffs [1]. Many studies have illustrated that overweight subjects are more emotionally anxious and more frequently resort to snacking compared to those of normal body mass [1, 7].

Obesity may be the source of stress that reinforces the obesity through increasing the body mass [9, 22], whilst on the other hand, dissatisfaction with one's body image may act as a spur to alter body mass [25]. This could explain why the attempt to diet is more frequent and adopting restriction diets is more common in obese women. Nevertheless, restriction diets can result in an excess body mass. When the constant attention to limiting food consumption becomes diverted, through falling prey to temptation, then overeating may occur [15]. Imposing dietary restrictions leads to the body's signals being ignored and a loss of being able to differentiate between being hungry or satiated [16, 32]. As aforementioned, obese and overweight women more often succumb to emotional overeating than the other subject groups. For this former group, the results thus both show the tendency for reducing dietary intake but increasing overeating for emotional reasons.

Scant attention is however directed towards those with a normal body mass, where it is assumed that this is a low risk group for overweight and obesity. The presented findings show that over half the normal body mass (weight) subjects would like to be a little slimmer and that 31% actively dieted. Studies on American female students have yielded similar results with 95% of those with a normal body mass wanting to lose weight. Changes in dietary behaviour in normal body mass women consisted of eating smaller than desired portions (44%) and replacing sugar with sweeteners by 31% [16]. Similar findings have been reported by *Burgic-Radmanovic* et al [5], who demonstrated that 50% of white Caucasian women of normal body mass wish to be underweight. *Jaworowska* and *Bazylak* [11] found that in students, only 34.4% of women were satisfied with their body mass and that these adopted restriction diets. Such diets can lead to future changes in one's body mass. The population Health Study of Nord-Trondelag (HUNT) showed that after the eleventh year of observation, teenagers who regarded themselves as obese, significantly increased their body mass much more than adults did [8]. Furthermore, subjects of normal body mass that perceived themselves as obese, had waists 3.46 cm larger than those who considered themselves to be not obese.

As has been shown, restriction diets are an inappropriate dietary behaviour and they do not prevent obesity. Such persons have a higher BMI and they have more difficulties in maintaining a correct body mass [29]. The nutritional behaviour of the subjects with normal body mass is also a cause for concern and requires further observation. In addition, with the substantial prevalence of poor body satisfaction, public health initiatives designed to improve body satisfaction along with promotion of healthy eating and active living. We recommend the present findings be confirmed in a longitudinal study among a large, population-based sample of young women.

CONCLUSIONS

1. The study found a relationship between subjective assessment of body weight and possessed body weight and eating behaviors, which should draw attention of physicians to the complexity of having abnormal body weight among young women.
2. It seems necessary to create a prevention and educational programs on proper nutrition and the perception of one's own body as effective tools in reducing eating disorders in terms of the health of young women and multigenerational inheritance health of their offspring.

Conflict of interest
The authors declare no conflict of interest.

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HOLISTIC MEASUREMENT OF WELL-BEING: PSYCHOMETRIC PROPERTIES OF THE PHYSICAL, MENTAL AND SOCIAL WELL-BEING SCALE (PMSW-21) FOR ADULTS

Piotr Supranowicz[1], Małgorzata Paź[2]*

[1]Department of Health Promotion and Postgraduate Education, National Institute of Public Health – National Institute of Hygiene, Warsaw, Poland
[2] Institute of Psychology, Faculty of Christian Philosophy, Cardinal Stefan Wyszyński University, Warsaw, Poland

ABSTRACT

Background. A holistic approach to health requires the development of tools that would allow to measure the inner world of individuals within its physical, mental and social dimensions.

Objectives. To create the Physical, Mental and Social Well-being scale (PMSW-21) that allows a holistic representation of various dimensions of well-being in such a way as they are perceived by the individuals and how affected their health.

Material and methods. The study was conducted on the sample of 406 inhabitants of Warsaw involving in the Social Participation in Health Reform project. The PMSW-21 scale included: headache, tiredness, abdominal pain, palpitation, joint pain, backache, sleep disturbance (physical domain), anxiety, guiltiness, helplessness, hopelessness, sadness, self-dissatisfaction, hostility (mental domain), security, communicability, protection, loneliness, rejection, sociability and appreciation (social domain). The five criterial variables of health and seven of life experiences were adopted to assess the discriminative power of the PMSW-21 scale.

Results. The total well-being scale as well as its physical, mental and social domains showed high reliability (Cronbach α 0.81, 0.77, 0.90, 0.72, respectively). The analysis confirmed the construct validity. All the items stronger correlated with their own domain than with the others (ranges for physical: 0.41 – 0.55, mental: 0.49 – 0.80 and social: 0.31 – 0.50). The total scale demonstrate high sensitivity; it significantly differentiated almost all criterial variables. Physical domain showed high sensitivity for health as well as for negative life events variables, while the mental and social domains were more sensitive for life events.

Conclusions. The analysis confirmed the usefulness of PMSW-21 scale for measure the holistic well-being. The reliability of the total scale and its domains, construct validity and sensitivity for health and life determinants were at acceptable level.

Key words: *holism, well-being, PMSW-21 scale, reliability, validity*

STRESZCZENIE

Wprowadzenie. Holistyczne podejście do zdrowia wymaga stworzenia narzędzia, które umożliwiłoby mierzenie wewnętrznego świata jednostki w jego fizycznym, psychicznym i społecznym wymiarze.

Cel. Opracowanie skali Fizycznego, Psychicznego i Społecznego Samopoczucie (PMSW-21), która umożliwi przedstawienie w sposób całościowy różnych wymiarów samopoczucia w taki sposób, jak są one postrzegane przez jednostki i jak wpływają na ich zdrowie.

Materiał i metody. Badania przeprowadzono na próbie 406 mieszkańców Warszawy biorących udział w projekcie Partycypacja Społeczna w Reformowaniu Zdrowia. Skala PMSW-21 obejmowała: ból głowy, przemęczenie, ból brzucha, kołatanie serca, ból stawów, ból pleców, trudności w zasypianiu (domena fizyczna), niepokój, poczucie winy, bezradność, beznadziejność, smutek, niezadowolenie z siebie, wrogość (domena psychiczna), bezpieczeństwo, komunikatywność, ochronę, samotność, wykluczenie, towarzyskość i szacunek (domena społeczna). Do oceny mocy dyskryminacyjnej skali PMSW-21 przyjęto pięć zmiennych kryterialnych dotyczące zdrowia i siedem dotyczących doświadczeń życiowych.

Wyniki. Zarówno całkowita skala, jak i jej domeny fizyczna psychiczna i społeczna wykazały wysoką rzetelność (Cronbach α odpowiednio 0.80, 0.77, 0.90, 0.72). Analiza potwierdziła trafność konstruktu. Wszystkie pozycje silniej korelowały z własną domeną niż z pozostałymi (zakresy dla fizycznej: 0.41 – 0.55, psychicznej: 0.49 – 0.80 i społecznej: 0.31 – 0.50). Całkowita skala wykazała wysoką czułość, znacząco różnicowała niemal wszystkie zmienne kryterialne. Domena fizyczna wykazała wysoką czułość zarówno w przypadku zmiennych kryterialnych dotyczących zdrowia jak i negatywnych zdarzeń życiowych, natomiast domeny psychiczna i społeczna były bardziej czułe w przypadku zdarzeń życiowych.

***Corresponding address:** Piotr Supranowicz, Department of Health Promotion and Postgraduate Education, National Institute of Public Health – National Institute of Hygiene, Chocimska Street 24, 00-791 Warsaw, Poland, tel. +48 22 54 21 334, fax +48 22 54 21 375, e-mail: psupranowicz@pzh.gov.pl

Wnioski. Analiza potwierdziła użyteczność skali PMSW-21 do całościowego mierzenia samopoczucia. Rzetelność skali całkowitej i jej domen, trafność konstruktu oraz czułość w odniesieniu do uwarunkowań zdrowotnych i życiowych była na akceptowalnym poziomie.

Słowa kluczowe: *holizm, samopoczucie, skala PMSW-21, rzetelność, trafność*

INTRODUCTION

The roots of the holistic theory of health seen as wholeness of external and internal components of human being go back to the ancient time. The Bible proclaims: "A glad heart is excellent medicine, a depressed spirit wastes the bones away" (The Proverbs, 17, 22) [16]. A holistic approach to health (from Greek "*holos*", meaning "all, whole, entire, total") has been more or less represented in the Western medicine since the time of Hippocrates. In the contemporary holistic medicine the body, mind and environment contribute equally to health and illness [3], what is in line with the WHO definition of health as " a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity" [28]. The interest in the holistic theory of health increased in the 70's of the twentieth century as an attempt to overcome the limitations of biophysical reductionism in medicine [11]. The *George Engel's* biopsychosocial model of health and its disorders was of great concern. *Engel* suggested that illness is commonly preceded by a period of psychological disturbances, during which the individual feels unable to copy. This has been designated the giving-up – given-up complex and has the following five psychological characteristics: a feeling of given-up, experienced as helplessness or hopelessness; a depreciated image of the self; a sense of loss gratification from relationships or roles in life; a feeling of disruption of the sense of continuity between past, present and future; and reactivation of memories of earlier period of giving-up [4]. Developing tools that would measure the inner world of the individual has become a challenge for researchers of holistic approach.

Until now a lot of the quality (or health-related quality) of life instruments were elaborated to explore subjective personal sphere, which may impinge on the health. The two of them, Short Form-Health Survey (SF-36) and World Health Organization Quality of Life (WHOQOL), were the most commonly used [2]. The SF-36 was the result of the research project of the Medical Outcome Study [25]. One hundred and forty nine items of the first version were subjected to exploratory factor analysis, which allowed to isolate 36 independent items that created the eight domains: physical functioning, role-physical, bodily pain, general health, vitality, social functioning, role-emotional, and mental health. The WHOQOL questionnaire was developed by experts from fifteen international centres. Of the 259 initially submitted items 100 were selected, which formed six dimensions: physical health, psychological health, level of independence, social relationship, environment, and spirituality [27]. Then, the construct validity was tested by confirmatory factor analysis. However, the both scales have serious disadvantages as holistic measures. Firstly, the domains consist of a different number of items, and therefore it does not allow to take into account all dimension of the well-being to the same extent. Secondly, the emphasis was placed on the objectification of measurement, what not always corresponds to the point of view of the individual.

Since 2001, the development of instruments for measuring well-being in its physical, metal and social dimensions, useful to determine the subjective circumstances of health, has been the object of research conducted in Department of Health Promotion and Postgraduate Education of the National Institute of Public Health – National Institute of Hygiene in Warsaw. The Physical, Mental and Social Well-being (PMSW18-Ad) scale for adolescents was elaborated and successfully applied in the international studies [8, 21-23]. The aim of presented paper is to examine psychometric properties of the Physical, Mental and Social Well-being scale (PMSW-21) for adults. Creating the scale, the following assumptions were made: 1) the scale allows a holistic representation of the various dimensions of well-being in such a way as they are perceived by the individual; 2) the scale creates the continuum, physical and social dimensions lie on its extremities, and mental dimension is the core; 3) the items co-creating the dimension correlate stronger with its own domain than with the others, nevertheless, they are also associated with the items of the other domains (acceptable skewness due to holistic nature of the scale).

MATERIAL AND METHODS

The sample consisted of 406 subjects living in Warsaw, who took part in the research project on social participation in health care reform in Poland. Characteristics of the sample and contents of questionnaire were presented in detail elsewhere [24].

The Physical, Mental and Social Well-being scale (PMSW-21) was developed in the Department of Health Promotion and Postgraduate Education of the National

Institute of Public Health – National Institute of Hygiene (NIPH-NIH) in Warsaw. The physical domain of the scale consisted of seven most commonly experienced ailments that are usually accompanied by various health disorders. The respondents were asked, how frequently, in general, they experience headache, tiredness, abdominal pain, palpitation, joint pain, backache and sleep disturbance. It was assumed that perceived severity of ailments will be measured in respect to the personal experience of subjects in general. Therefore, the relative frequency of ailments was registered on five-points scale from 'very often' (1 point) to 'very rarely or never' (five points). The overall physical domain ranged from 7 points to 35 points, and the higher scores indicated the better physical well-being. The similar procedures were used for constructing the mental domain of the scale. This domain contained seven items concerning feelings and emotions that, if had been frequently experienced or in a long period, they were identified as risk factors for stress-related diseases or mental disorders, namely: anxiety, guiltiness, helplessness, hopelessness, sadness, self-dissatisfaction and hostility. The social domain of the scale also consisted of seven items. The subjects were asked to what extent they agree with the statements included in the questionnaire. The statements concerned (statements in parentheses): security ('I feel safe in my everyday life'), communicability ('Contacts with other people are often difficult for me'), protection ('I can rely on the help from relatives'), loneliness ('I often feel lonely'), rejection ('People often criticise me'), sociability ('I like to be with people') and appreciation ('I feel appreciated by people'). The subjects could choose one of five responses from 'definitely not' (1 point) to 'definitely yes' (5 points). The variables based on negative formulated statements (communicability, loneliness and rejection) were recoded in such a way that all items of the social well-being domain were measured in the same direction. The social well-being domain also ranged from 7 points to 35 points, and the higher scores designated the better social well-being. The overall well-being scale were the sum of the three domains and ranged from 21 to 105 points.

The health indicators and negative life events were assumed as criteria for validity assessment of PMSW-21 scale. The health indicators measured: self-rated health (very good or good / not good), staying at home in the previous year due to illness (never / at least one time), consulting the physicians in the previous year (0-1 time / more than 1 time), occurrence of chronic disease (none / at least 1 chronic disease) and hospitalisation in the previous year (never / at least one time). Furthermore, the subjects were asked, weather they experienced negative life events in the previous year, and seven of the most commonly events were included: family problems, financial difficulties, lack of opportunities for relaxation, problems at workplace, difficult house conditions, encountering with violence and restriction in social contacts.

The Epi Info program was applied for creating the database and statistical analysis. The reliability was measured by Cronbach α coefficient for internal consistency, according to the formula [14]:

$$\alpha=k/k\text{-}1(1-\Sigma s_i^2/s_t^2),$$

where:
k – number of items in domain, Σs_i^2 – sum of item variances, s_t^2 – variance of total domain. The *Nunnaly* criterion of reliability $\alpha>0.7$ was accepted [17].

Due to initial assumption of belonging of individual items to hypothetical domains, the analysis of construct validity was of nature of confirmatory analysis. The fit of construct was analysed by examining the convergent, divergent and structural validity of the domains of the PMSW-21 scale. The convergent validity was shown by the mean correlation between the items of the same domain, while divergent validity was identified by the mean correlation between the items of different domains. The structural validity confirms the contribution of particular items to the hypothetical domain. *Pearson's* coefficient of correlation was used to measure the relationship between variables. Strength of correlation was interpreted in accordance with the general accepted convention [7]: $0.1\geq |r|$ – lack of correlation, $0.1< |r| \leq 0.3$ – weak correlation, $0.3 < |r| \leq 0.5$ – moderate correlation, $0.5 < |r| \leq 0.7$ – high correlation, $0.7< |r| \leq 0.9$ – very high correlation, $0.9< |r|$ – almost all identity. With regards to the correlation between the scale items, it was assumed that correlation $r>0.80$ indicates that the both items measure the same phenomenon (the level of redundancy) [18]. Moreover, the items are expected to correlate with their domains at least at the level $r=0.40$.

The discriminant validity of PMSW-21 scale in relation to health and life determinants was examined by *Mann-Whitney* test. The term 'discriminant validity' was usually used for defining the external criterion of validity of tested instruments to identify the expected differences between the distinct groups of subjects [6, 20]. It should be noted, however, that 'discriminant validity' was also used interchangeably with 'divergent validity' [10, 12].

The significance was accepted at the level $p<0.05$.

RESULTS AND DISCUSSION

Comparing the level of well-being the respondents perceived, the social domain was assessed the hi-

ghest, while physical domains was considerably lower (Table 1). The SF-36, WHOQOL and many other quality of life scales have a different number of items of each domain, therefore, it is difficult to compare the various dimensions of health among themselves. However, the large differences occurred between the countries in the scores of all domains, for example in the international study of psychometric properties of WHOQOL scale the ranges of domains for 23 countries were: 12.1 – 17.1 for physical, 10.6 – 15.4 for psychological, 10.8 – 15.8 for social and 10.7 – 15.9 for environmental domain [20].

Table 1. Descriptive statistics and reliability of the PMSW-21 scale and its domains

PMSW-21	Descriptive statistics			Reliability
	Mean	(SD)	Range	Cronbach α
Total	74.4	(12.7)	35 – 103	0.81
Domains:				
Physical	22.6	(5.6)	7 – 35	0.77
Mental	24.8	(6.6)	7 – 35	0.90
Social	27.1	(4.2)	11 – 35	0.72

The PMSW-21 scale and its domains demonstrated the reliability at the acceptable level (Table 1). The mental domain has substantially higher reliability. The quality of life scales commonly used, such as SF-36 or WHOQOL, mostly showed the high internal consistency, nevertheless, in many cases it was not confirmed for certain domains. In the *McPherson* and *Martin* review of literature on SF-36 psychometric properties only physical functioning and role physical domains were found of high reliability (α ranged 0.80 – 0.98 and 0.75 – 1.00, respectively), while differences in reliability of vitality, social functioning and mental health domains were large (α ranged 0.28 – 0.88, 0.30 – 0.98 and 0.39 – 0.90, respectively) [15]. The level of reliability of certain domains may considerably differ between the distinct population, for example healthy and disabled people [6, 9] or sufferers from various diseases [13].

The analysis of convergent, divergent and structural validity confirms the satisfactory construct organising of the PMSW-21 scale. Each item correlates stronger with the items of its own domain than with those of the other domains (Table 2 and 3). The differences are substantially higher in the mental domain items, while in three items of the social domain (security, loneliness and rejection) they are small. The four items (joint pain, backache, hopelessness and hostility) demonstrate orthogonality, the ranges of the remaining items overlap in part, and it is on line with our assumption of a partial skewness of the domains. None of the correlations exceeds the accepted limit (the highest correlation between helplessness and hopelessness is r=0.80), what indicates the lack of redundancy in any item (Table 2). Moreover, all items stronger correlate with their domain than with the others, however, the three items, tiredness from phy-

Table 2. The correlation between items of PMSW-21 scale

Items[1]	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.	20.	21.
1. headache	-																				
2. tiredness	**0.39**	-																			
3. abdominal pain	**0.40**	**0.30**	-																		
4. palpitation	**0.23**	**0.35**	**0.31**	-																	
5. join pain	**0.21**	**0.23**	**0.21**	**0.34**	-																
6. backache	**0.23**	**0.36**	**0.22**	**0.32**	**0.53**	-															
7. sleeping trouble	**0.24**	**0.35**	**0.29**	**0.41**	**0.33**	**0.37**	-														
8. anxiety	0.29	0.39	0.19	0.38	0.14	0.17	0.35	-													
9. guiltiness	0.18	0.32	0.10	0.21	0.05	0.09	0.21	**0.64**	-												
10. helplessness	0.26	0.43	0.24	0.34	0.14	0.19	0.29	**0.66**	**0.58**	-											
11. hopelessness	0.27	0.39	0.20	0.28	0.14	0.20	0.28	**0.63**	**0.52**	**0.80**	-										
12. depression	0.24	0.45	0.19	0.30	0.15	0.22	0.35	**0.66**	**0.60**	**0.70**	**0.78**	-									
13. self-dissatisfaction	0.13	0.30	0.07	0.22	0.07	0.13	0.22	**0.54**	**0.63**	**0.59**	**0.59**	**0.64**	-								
14. hostility	0.06	0.20	0.11	0.16	0.05	0.09	0.15	**0.35**	**0.36**	**0.40**	**0.44**	**0.42**	**0.48**	-							
15. security	0.24	0.27	0.10	0.18	0.06	0.11	0.18	0.41	0.29	0.39	0.39	0.38	0.30	0.15	-						
16. communicability	0.20	0.09	0.08	0.12	0.03	0.05	0.05	0.31	0.28	0.30	0.34	0.30	0.33	0.22	**0.22**	-					
17. protection	0.09	0.09	0.09	0.14	0.00	0.08	0.13	0.17	0.09	0.27	0.26	0.22	0.20	0.09	**0.38**	**0.15**	-				
18. loneliness	0.24	0.27	0.13	0.19	0.14	0.16	0.23	0.40	0.38	0.41	0.44	0.51	0.41	0.19	**0.37**	**0.36**	**0.36**	-			
19. rejection	0.14	0.23	0.10	0.19	0.06	0.09	0.09	0.11	0.19	0.17	0.17	0.15	0.23	0.19	**0.12**	**0.23**	**0.11**	**0.25**	-		
20.. sociability	0.03	0.02	-0.03	0.00	-0.04	-0.02	0.00	0.18	0.15	0.17	0.25	0.17	0.24	0.21	**0.25**	**0.33**	**0.22**	**0.22**	**0.25**	-	
21. appreciation	0.01	0.07	-0.01	-0.01	-0.12	-0.04	0.01	0.17	0.13	0.27	0.27	0.18	0.26	0.15	**032**	**0.25**	**0.41**	**0.29**	**0.22**	**0.40**	-

[1] *Pearson's* coefficient of correlation, $p<0.05$ if $|r|>0.10$; the bold print indicates the correlation with the scale that an item co-creates.

Table 3. Convergent and divergent validity of PMSW-21 scale

Items[1]	Convergent validity		Divergent validity	
	r (mean)	range	r (mean)	range
Physical domain:				
Headache	0.29	0.21 – 0,40	0.17	0.01 – 0.29
Tiredness	0.33	0.23 – 0.40	0.25	0.02 – 0.45
Abdominal pain	0.29	0.21 – 0.35	0.11	-0.03 – 0.24
Palpitation	0.33	0.21 – 0.41	0.19	-0.01 – 0.38
Join pain	0.31	0.27 – 0.53	0.06	-0.12 – 0.15
Backache	0.35	0.24 – 0.53	0.11	-0.04 – 0.22
Sleep disturbance	0.33	0.24 – 0.41	0.18	0.00 – 0.35
Mental domain:				
Anxiety	0.58	0.35 – 0.66	0.26	0.11 – 0.40
Guiltiness	0.56	0.36 – 0.64	0.19	0.05 – 0.38
Helplessness	0.62	0.40 – 0.80	0.27	0.14 – 0.43
Hopelessness	0.63	0.44 – 0.80	0.28	0.14 – 0.44
Depression	0.63	0.42 – 0.78	0.27	0.15 – 0.51
Self-dissatisfaction	0.57	0.48 – 0.64	0.22	0.07 – 0.41
Hostility	0.41	0.35 – 0.48	0.14	0.05 – 0.21
Social domain:				
Security	0.28	0.12 – 0.38	0.24	0.06 – 0.41
Communicability	0.26	0.15 – 0.38	0.19	0.03 – 0.34
Protection	0.27	0.11 – 0.41	0.14	0.00 – 0.27
Loneliness	0.31	0.20 – 0.37	0.29	0.13 – 0.51
Rejection	0.18	0.11 – 0.25	0.15	0.06 – 0.23
Sociability	0.28	0.20 – 0.40	0.10	-0.04 – 0.25
Appreciation	0.32	0.22 – 0.41	0.10	-0.12 – 0.27

[1]*Pearson's* coefficient of correlation, $p<0.05$ if $|r|>0.10$

Table 4. Structural validity of PMSW-21 scale

Items[1]	Domains:		
	Physical	Mental	Social
Physical:			
headache	**0.41**	0.22	0.25
tiredness	**0.48**	0.45	0.27
abdominal pain	**0.42**	0.18	0.11
palpitation	**0.50**	0.34	0.22
joint pain	**0.48**	0.10	0.03
backache	**0.55**	0.18	0.10
sleeping disturbance	**0.51**	0.31	0.18
Mental:			
anxiety	0.42	**0.72**	0.43
guiltiness	0.23	**0.69**	0.36
helplessness	0.42	**0.79**	0.47
hopelessness	0.40	**0.79**	0.50
sadness	0.43	**0.80**	0.45
self-dissatisfaction	0.25	**0.72**	0.47
hostility	0.19	**0.49**	0.27
Social:			
security	0.30	0.43	**0.45**
communicability	0.17	0.36	**0.43**
protection	0.16	0.24	**0.45**
loneliness	0.33	0.47	**0.50**
rejection	0.21	0.21	**0.31**
sociability	0.01	0.25	**0.43**
appreciation	0.02	0.25	**0.51**

[1]*Pearson's* coefficient of correlation, $p<0.05$ if $|r|>0.10$
The bold print indicates the correlation with the domain that an item co-creates (an item was excluded from the domain to protect overlap).

Table 5. Correlation between the domains of PMSW-21 scale

Domains[1]	Physical		Mental	
	r	r^2	r	r^r
Physical	-	-		
Mental	0.40	0.16	-	-
Social	0.29	0.08	0.53	0.28

[1] r – *Pearson's* coefficient of correlation, $p<0.05$ if $|r|>0.10$
r^2 – coefficient of determination.

sical domain, and security and loneliness from social domain, correlate with mental domain only slightly weaker (Table 4). Almost all items (except rejection) correlate at the level $r>0.40$. The strength of correlations of the mental items with their domain are very high, whereas those of the remaining domains were high or moderate. As regards the relation between the domains, the metal health correlates moderately or high with both the other domains, while correlation between physical and social domains is weak (Table 5), what confirms that mental domain is a core of the PMSW-21 scale. The study verifying the content validity of the life quality scales yielded inconsistent results. Analysing the convergent and divergent validity of the Lithuanian WHOQOL, *Baceviciene* et al. found the correlations between items inside the designated domain were considerably stronger then those with the items of the other domains [1]. In contrast, in the Brazilian version of SF-36 the ranges

Table 6. Discriminant validity of PMSW-21 scale in relation to health and life determinants

Determinants[1]	Total		Domains: Physical		Mental		Social	
	X	p	X	p	X	p	X	p
Health								
Self-rated health		<0.001		<0.001		0.079		0.001
very good or good	78.4		25.5		25.4		27.9	
not good	70.6		20.0		24.2		26.4	
Staying at home due to illness		0.038		0.002		0.007		0.506
never	76.0		23.7		25.8		27.2	
at least 1 time	73.1		21.7		23.9		27.0	
Physician consultation		0.030		0.243		0.029		0.859
0-1 time	76.2		23.0		25.7		27.1	
more than 1 time	73.2		22.3		24.1		27.0	
Chronic disease		<0.001		<0.001		0.809		0.092
none	79.0		25.5		25.1		27.6	
at least 1 disease	73.2		21.8		24.7		26.9	
Hospitalisation		0.646		0.025		0.568		0.019
never	74.2		22.9		24.7		26.8	
at least 1 time	75.1		21.5		25.2		27.9	
Negative life events								
Family problems		0.006		0.011		0.001		0.049
no	76.1		23.4		25.8		27.5	
yes	72.6		21.7		23.7		26.6	
Financial difficulty		<0.001		0.004		<0.001		0.003
no	76.6		23.3		25.8		27.5	
yes	70.9		21.4		23.1		26.2	
Lack of opportunity for relaxation		<0.001		0.001		<0.001		<0.001
no	77.5		23.4		26.3		27.8	
yes	70.2		21.4		22.6		25.9	
Problems at workplace		0.006		0.412		<0.001		0.028
no	75.6		22.8		25.5		27.3	
yes	71.7		22.1		23.0		26.4	
Difficult house conditions		0.443		0.737		0.054		0.623
no	74.4		22.6		25.0		27.1	
yes	73.8		22.2		23.1		26.8	
Encountering with violence		0.003		0.002		0.031		0.007
no	75.1		22.9		25.0		27.3	
yes	67.0		19.2		22.4		24.8	
Restriction in social contacts		<0.001		<0.001		<0.001		<0.001
no	77.3		23.4		26.1		27.9	
yes	67.5		20.7		21.8		25.2	

[1]*Mann-Whitney* test

of correlation inside the domain and with the items of other domains overlapped in three out of eight domains [12]. Also confirmation of structural validity of the life quality scales varied between the studies. The Lithuanian version of WHOQOL presented the remarkably higher correlations between the items and their own domain than with other domains, although in many cases the letter correlations were significant too [1]. On the other hand, in the Chinese WHOQOL version 20% of items correlated stronger with the domains other than hypothesized [13]. This inconsistency in construct assumptions was observed also in some country versions of the SF-36 [5, 19].

The total PMSW-21 scale demonstrated high sensitivity in relation to both the health and life event factors (Table 6). Significant differences were observed in almost all criterial indicators (except hospitalisation and difficult house conditions). Physical domain showed high sensitivity for health as well as life event indicators, while the mental and social domains were more sensitive for negative life events. It is interesting that hospitalised patients felt worse physically, while they simultaneously perceived better the social support. This would suggest that hospitalisation may provide a sense of security, and also demonstrates the complexity of the components of well-being. The previous studies on quality of life examined the discriminant validity using healthy and sick or disabled samples. The international comparative analysis of sensitivity of WHOQOL found differences between the countries. Out of 14 countries,

the sensitivity for physical domain did not confirmed in 3 countries, for psychological domain – in 4 countries, and for social domain – in 6 countries [20]. The comparison of WHOQOL of mentally and physically ill revealed the significant differences in psychological and social, but not physical domains [6]. The sensitivity analysis of the SF-36 showed a weak correlation between domains and criterial variable, which was the changes in health over the last year [26].

CONCLUSIONS

Our findings confirmed the usefulness of PMSW-21 scale for holistic measure of well-being. In particular:

1. The reliability of the total scale as well as its domains was at acceptable level.
2. The construct validity met initial assumptions; the particular items showed the great fit for the intended domains, and the correlations within and with the mental domain (core) was the highest.
3. The total scale and its domains presented the high sensitivity in relation to different determinants of well-being (health and life experiences).

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Conflict of interest

The authors declare no conflict of interest.

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