Vol. 17, No. 1 2010

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HYPHENATED AND UNCONVENTIONAL METHODS FOR SEARCHING VOLATILE CANCER BIOMARKERS

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Abstract: Volatile organic compounds produced inside the body provide valuable information about human state of health and they are detected in breath, blood and urine samples. Therefore, volatile biomarker analysis seems to become accurate and fast method for tumour detection. So far, there are known several volatile organic compounds (VOCs) recognized as potential cancer biomarkers. For the detection of VOCs different analytical techniques are used. The most popular is gas chromatography coupled with mass spectrometry (GC/MS). More recently, selected ion flow tube mass spectrometry (SIFT-MS), proton transfer reaction mass spectrometry (PTR-MS) and ion mobility spectrometry (IMS) are also applied for biomarker research. Besides typical instrumental methods used for VOCs analysis, unconventional methods such as sensitive canine sense of smell can be used. In recent years, this very sensitive scent is also used for cancer biomarker detection. Dogs are trained to recognize the smell of skin, breath or urine samples from patient with different kind of cancer from the control group. The application of dogs' smell for the preliminary screening of tumour in human body is painless, noninvasive and fast method. Additionally, it does not need the preconcentration of analytes before the analysis.

Keywords: analytical methods, canine scent, biomarkers, volatile organic compounds, cancer

Introduction

Cancer diseases are leading a few million deaths worldwide every year. Early detection of tumour increases the chance to survive. So far, there are known many different methods used for tumour diagnosis. The most popular is computer tomography, nuclear magnetic resonance, positron emission tomography, mammography and single photon emission computed tomography [1]. However, these methods are expensive and could be harmful for patients. Biomarker analysis is complementary method and seems to be screening method for early cancer detection. Molecular biomarker is defined as a molecule which reflects the

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pathological state of the organism and it can be characteristic pharmacologic response to a therapeutic intervention. The expression of estrogen receptors (ER), progesterone receptor (PR), p53, B-cell lymphoma-2 (Bcl-2), cyclin E, cytokeratin 5/6, human epidermal growth factor-2 (HER-2/neu) oncogenes and the Ki-67 index of proliferative activity are protein biomarkers used in medicine [2, 3]. However, huge number of various classes of compounds present in the tissue makes protein biomarkers searching extremely difficult. Therefore, analysis of substances present in exhaled breath seems to be easier to perform, faster and non-invasive method which can be used for cancer detection [4, 5].

In exhaled air more than 2000 different compounds can be detected [6]. These substances are in the most cases volatile organic compounds (VOCs) but nonvolatile organic compounds (proteins, leukotrienes, etc.) and inorganic volatile compounds (carbon oxide, nitrogen oxides, carbonyl sulfide) are also identified. Their exhaled amount may change depends on age, state of health, life style, gender. However, in pathological states of the organism some additional compounds are produced and/or their concentration is changed [7].

Gas chromatography with flame ionization detector (FID) or with mass spectrometry (MS) has been applied for detection and quantitation compounds existing in breath. [8, 9]. Different mass spectrometry techniques such as proton-transfer-reaction mass spectrometry (PTR-MS) [10], selected-ion-flow-tube mass spectrometry (SIFT-MS) [11], ion mobility spectrometry (IMS) [12] allow to on-line breath testing.

Except analytical methods, canine sense of smell can be use for cancer detection [13]. At first, dog's olfactory system was used in finding bombs, drugs and people [14]. Nowadays, trained dogs smelling different probes (ie skin, urine, breath) are able to recognize samples from people with cancer and healthy volunteers.

The aim of this review is the presentation of instrumental analytical methods and canine scent which can be used for detection of volatile cancer biomarkers. Substances recognized as potential biomarkers have been also present.

Volatile organic compounds proposed as cancer biomarkers

Volatile organic compounds present in exhaled air have both origins - endogenous or exogenous. First are produced during different biochemical processes which occur in living organisms and second can be undertaken with food, inhaled with the air or absorbed by the skin. Only the first group can be considered as a diseases marker. There are known metabolic pathways of formation only limited examples of endogenous substances. Saturated hydrocarbons (ethane, pentane) and aldehydes (acetaldehyde) are generated during lipid peroxidation of fatty acids (Fig. 1) [5]. According to Spanel et al formaldehyde is a potential biomarker of bladder and prostate cancer [15]. Concerning to another aldehydes such as heptanal and hexanal they are supposed to be characteristic for patients with breast or lung cancer [16-21]. Acetone is one of the most abundant compound in breath and an important metabolic marker in breath [22]. It is ultimately formed by the decarboxylation of acetoacetate, which is derived from lipolysis or lipid peroxidation (Fig. 2). Acetone has been recognized by Phillips et al [18] as a potential marker of lung cancer. Isoprene (2-methyl-1,3-butadiene) is a hydrocarbon always present in breath which is a by-product of cholesterol synthesis [23] (Fig. 3). Poli et al found that in breath of patients with lung cancer concentration of isoprene was higher than in control group.

Volatile organic compounds chosen as hypothetical cancer biomarkers are summarized in Table 1.

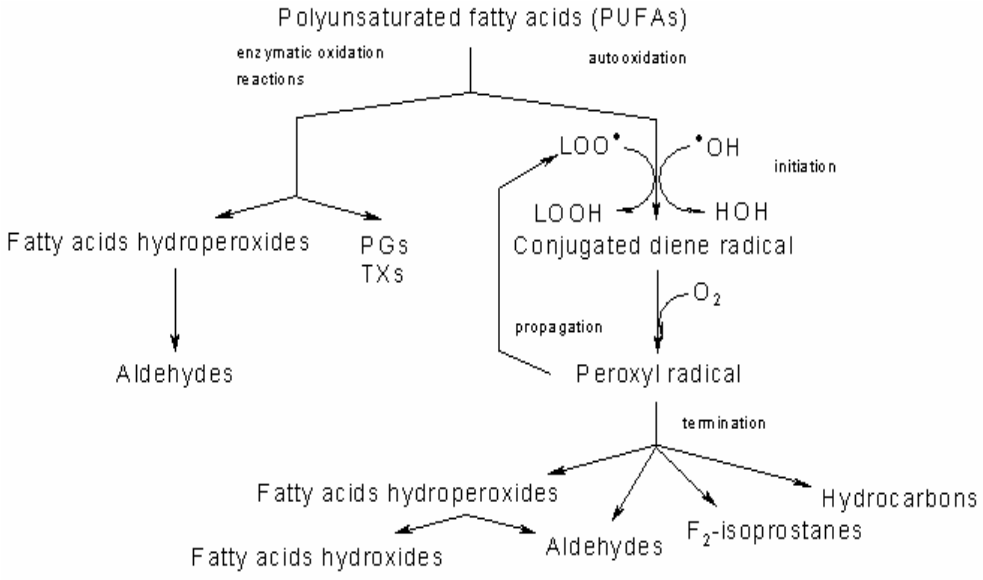

Fig. 1. Products generated in polyunsaturated fatty acids oxidation [4]

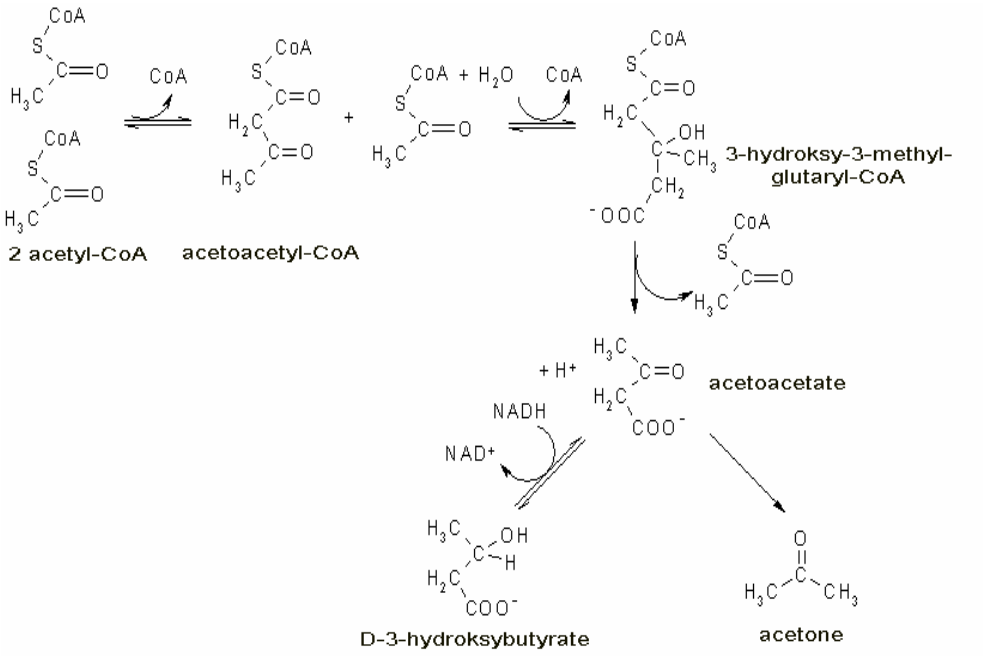

Fig. 2. Biochemical pathway of acetone generation with excess of acetyl-CoA [24]

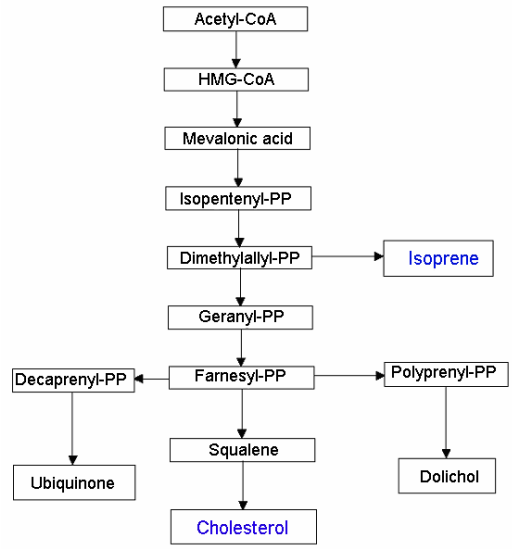

Fig. 3. Biochemical pathway of isoprene generation [5]

Table 1

Volatile organic compounds recognized as cancer biomarkers

Compound	Type of cancer	Applied technique for VOCs analysis	Concentration level	References
isoprene	lung	SPME-GC/MS SPME-GC/FID	[ppb/ppt] [ppm]	[14, 15]
pentane	lung	SPME-GC/MS	[ppb/ppt]	[14]
octane	lung	SPME-GC/MS	[ppb/ppt]	[14]
decane	lung	SPME-GC/MS SPME-GC/FID	[ppb/ppt] [ppm]	[14, 15]
undecane	lung	SPME-GC/FID	[ppm]	[15]
methylcyclopentane	lung	SPME-GC/FID	[ppm]	[15]
formaldehyde	bladder, prostate, lung	PTR-MS	[ppb]	[17, 18]
heptanal	lung, breast	SPME-GC/FID SPME-GC/MS TD-GC/MS	[ppm] [ppb/ppt] [ppt]	[15, 19-22]
hexanal	lung, breast	SPME-GC/FID SPME-GC/MS TD-GC/MS	[ppm] [ppb/ppt] [ppt]	[15, 19-22, 26]
acetone	lung	TD-GC/MS SPME-GC/MS	[ppt] [ppb/ppt]	[20, 24]
2-propanol	breast	TD-GC/MS	[ppt]	[22]
benzene	lung	SPME-GC/MS SPME-GC/FID	[ppb/ppt] [ppm]	[14, 15]
toluene	lung	SPME-GC/MS	[ppb/ppt]	[14]
xylene isomers	lung	SPME-GC/MS TD-GC/MS	[ppb/ppt] [ppt]	[14, 20, 21]
ethylbenzene	lung	SPME-GC/MS	[ppb/ppt]	[14]

Analytical methods for detection of biomarkers

According to the requirements and biochemical properties of analyzed compounds, different analytical methods can be used for volatile biomarkers analyzes (Tab. 1). Some of them such as GC/MS are usually applied for separation and identification of unknown substances present in gaseous samples. Others, ie PTR-MS or SIFT-MS are often used for on-line monitoring of target compounds present in breath air.

Gas chromatography with mass spectrometry (GC/MS)

Gas chromatography is one of the most important analytical methods in organic chemical analysis for determination of individual substances in mixture. Mass spectrometry became an indispensable detector for GC because of selectivity, high sensitivity and identification potential.

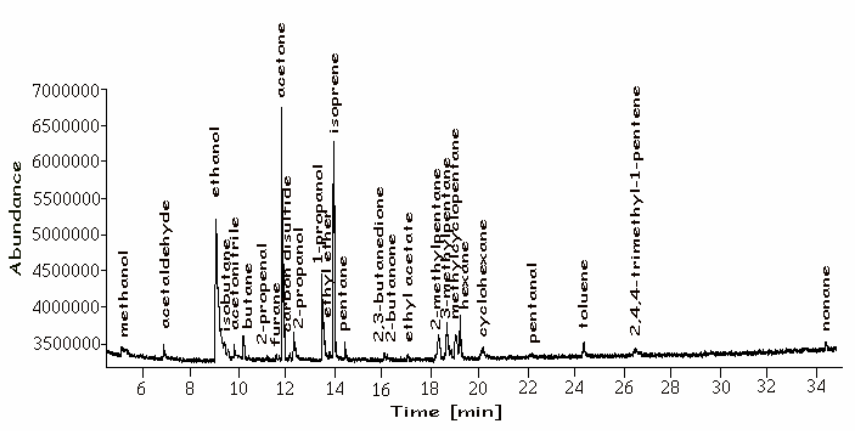

Fig. 4. The exemplary GC/TOF-MS chromatogram of exhaled air from healthy volunteer

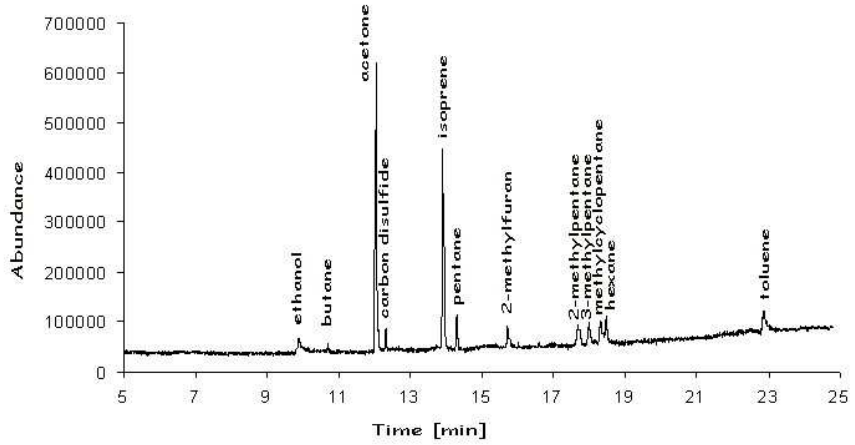

Fig. 5. The exemplary GC/MS chromatogram of exhaled air from healthy volunteer

Therefore, GC/MS technique is still gold standard in breath analyses [25-27] and gaseous emission from lung cancer cell lines at ppb or even ppt level [21]. Nowadays, GC combined with time of flight analyser (GC/TOF-MS) becomes forceful for identification of complicated gaseous mixture. The exemplary GC/TOF-MS and GC/MS chromatograms of exhaled breath of healthy persons are presented in Figure 4 and Figure 5, respectively. Due to very low concentrations of VOCs (ppb-ppt) existing in breath samples the preconcentration techniques such as trapping on solid sorbents followed by thermal desorption (TD) or solid-phase microextraction (SPME) are used prior chromatographic analysis [21, 27].

Gas chromatography with flame ionization detector (GC/FID)

Due to simplicity and high sensitivity the flame ionization detector is often using for breath analysis. The application of GC/FID system allows to detect hydrocarbons and their derivatives at ppm even ppb level (Tab. 1). This detector was applied for determination of acetone, pentane, isoprene, hexanal and others VOCs present in the breath samples [9, 16]. Chen et al applied GC/FID technique for determination of decane, isoprene, benzene, hexanal and heptanal in the headspace of lung cancer cells [16]. Isoprene and sulfur related compounds released by bacteria cultures were analyzed by Schfller et al with using this technique [28].

Proton transfer reaction mass spectrometry (PTR-MS)

The proton transfer reaction mass spectrometry is the technique appropriate for rapid and on-line measurements of VOCs present in human breath [29]. Some of volatile compounds can be detected in ppb concentration level.

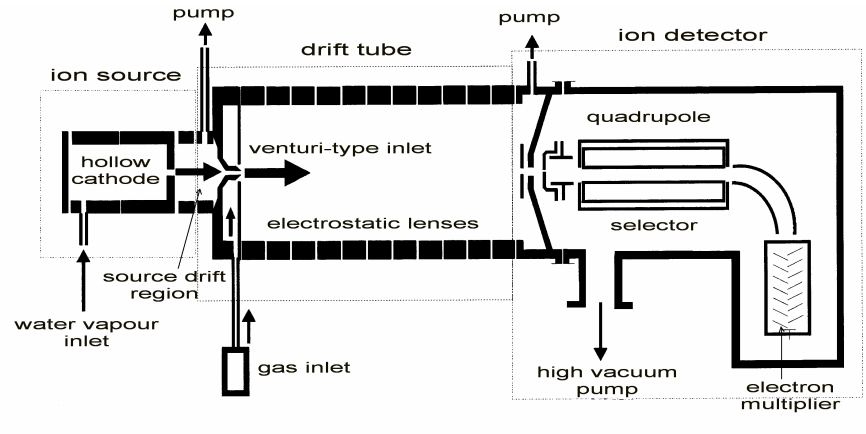

Fig. 6. Scheme of separation system in PTR-MS [30]

The basic principle of PTR-MS involves the mixing of a flowing air sample in a drift tube equipped with a source of H_3O^+. Ionization of vapour water can be done with using corona discharge, electron impact or alpha radiation emitted by ^{241}Am. Analytes rapidly react with reactant ions. During proton transfer reactions protonated cations of analyzed

substances are formed which then are separated in electric field according their mass to charge ratio (*m/z*). Identification in PTR-MS is based on molecular mass and thus it is possible interferences from various molecular species to appear. The scheme of PTR-MS separation system has been shown in Figure 6. Therefore, this method should be used for monitoring the concentration of analyte rather than for mixture separation [30]. This method was used to analyze breath isoprene with no preconcentration or preseparation [31] and for determination of acetonitrile and benzene concentration in smokers' breath samples [32].

Selected ion flow tube mass spectrometry (SIFT-MS)

Selected ion flow tube mass spectrometry is used for rapid detection and quantification of trace gases [33]. SIFT-MS involves the chemical ionization of trace gases introduced into flow tube using selected precursor positive ions such as H_3O^+, NO^+ and O_2^+ [34]. However, the most commonly used precursor ion is H_3O^+ which reacts with a wide range of organic species. In this technique sample of VOCs is added to the carrier gas/precursor ion swarm and the trace gases in the sample react with the precursor ions generating characteristic protonated product ions. Then mass spectrometer measures the count rates of the precursor and product ions. The quantification of particular trace gases in the air sample is achieved. Figure 7 presents the scheme of SIFT-MS system. SIFT-MS technique requires minimum sample preparation and no separation of analytes. It was used for on-line analysis of acetone, isoprene, ammonia and acetonitrile present in exhaled breath of smokers and nonsmokers [33, 35] and for analysis of formaldehyde in the headspace of urine from bladder and prostate cancer patients [15].

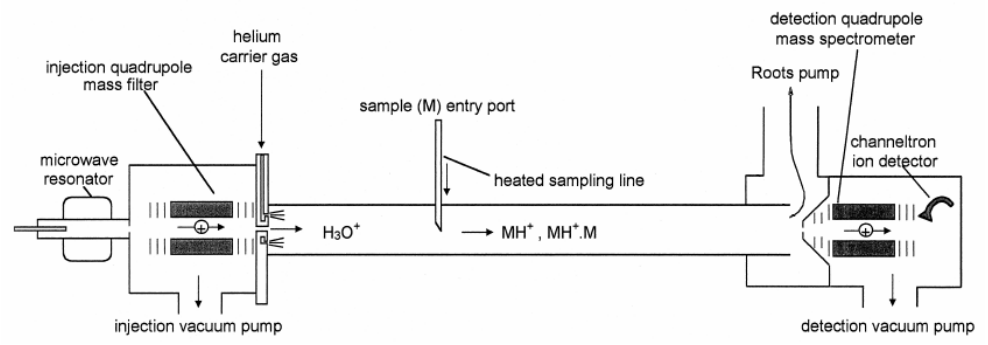

Fig. 7. Scheme of separation system in SIFT-MS [33]

Ion mobility spectrometry (IMS)

Ion mobility spectrometry is very effective and sensitive technique for the determination traces of aldehydes, ketones and esters in low ppb/ppt range [36, 37]. The time required to acquire a single spectrum is in the range of 20÷50 ms. There is no vacuum required for IMS operation and the ambient air can be used as carrier gas. Therefore, the IMS can be miniaturized and applied as an on-line technique for breath detection. Working principles of this device have been described in details by Baumbach [36]. IMS shows low sensitivity in case of alkanes and benzene-related compounds - analytes with the low proton

affinity. It is often coupled with standard gas chromatographic columns or multicapillary column (MCC) which enables analysis mixture of gaseous substances on-line and in very short time [38]. The MCC-IMS system was used for on-line breath analysis and compounds such as acetone, ammonia and ethanol were determined [39]. Scheme of typical drift tube used in IMS is presented in Figure 8.

The main advantages of PTR-MS, SIFT-MS and IMS are high sensitivity, possibility of VOCs determination without preconcentration process and on-line analysis.

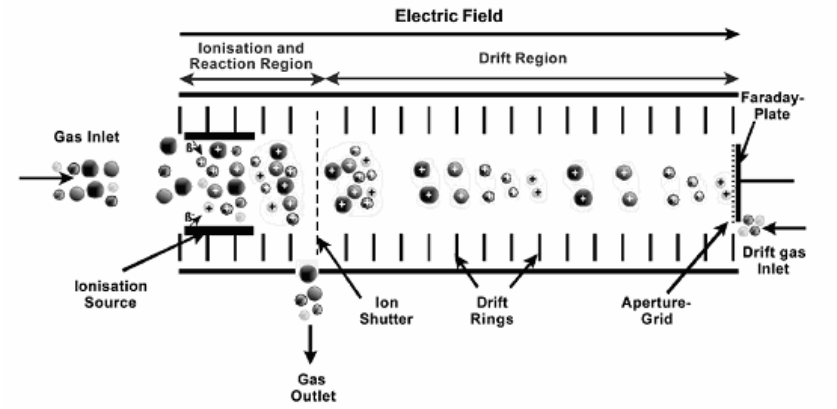

Fig. 8. Scheme of drift tube in ion mobility spectrometer [36]

Sensors

Sensors are become more popular in breath tests such as ammonia, ethanol, NO, CO detection [40]. These systems are relatively cheap, easy to provide and fast response. There is now great interest in the clinical application of an electronic nose; that it is possible for e-nose to diagnose different illness [41, 42]. Different types of sensors are used: bulk acoustic wave sensors [43], surface acoustic wave sensors [44], and multiple conducting polymer sensors [45]. A novel type of gas sensor array was used for quick self-check of breath of malodor components like ammonia, hydrogen sulfide and methanethiol [46]. Another type of sensor is e-nose, named Cyranose C320, based on multiple conducting polymer sensor technology. It has been used to distinguish the breath of smokers from that of non-smokers [47]. The advantage of the sensor is that presence of humidity that not has any influence on the outcome of analysis. Nitrogen oxide/dioxide measurement sensor system is used for on-line recognition of complex VOCs mixtures from patients with asthma [48].

Canine scent

According to Walker et al dog's sense of smell is estimated to be 10,000 times more sensitive than human, that means they have from 20 to 40 times more nasal receptor cells than human [49]. Figure 9 shows smelling process by dogs. This ability for many years has been used in finding bombs, drugs, and people [14]. From 1989, when William and Pembroke published the letter in *Lancet* [50] and described a case of woman seeking

medical attention because of her dogs' continued interest in a skin lesion, scientists decide to use canine scent for cancer detection.

Many analytical investigations confirmed that tumour cells produce volatile chemicals [21] that can be detected in breath [51], urine [52], blood [53] or emitted through the skin [54]. A different smell of probe sampled from healthy and ill persons enable recognizing them by dogs. Nowadays, dogs are trained to detect a various type of cancer by smelling urine [13, 55, 56], breath [57] and skin samples [58]. Five dogs were trained to distinguish, by scent, exhaled breath samples of 55 lung and 31 breast cancer patients from those of 83 healthy controls [57]. Horvath et al taught the dog to distinguish different histopathological types and grades of ovarian carcinomas, including borderline tumours, from healthy control samples [55]. For the experimenters, recruited dogs, should reveal high level of eagerness to sniff objects and respond to commands [57]. Canine scent of six trained dogs has been used to discriminate between urine from patients with bladder cancer and urine from diseased and healthy controls [56]. Willis et al achieved the successful detection of urine samples from patients with bladder cancer 41%. Multivariate analysis suggests that the dogs' capacity to recognize an odour signature characteristic of bladder cancer is independent of other chemical aspects of the urine detectable by urinalysis, such as the presence of blood. Melanoma is the skin cancer which can be first aided by visual inspection by the physician and then confirmed with histopathological research. Another way for its detection is finding the source of chemical markers with using the canine olfaction [59]. Two dogs with satisfactory results were trained to localize melanoma and recognize it from healthy skin.

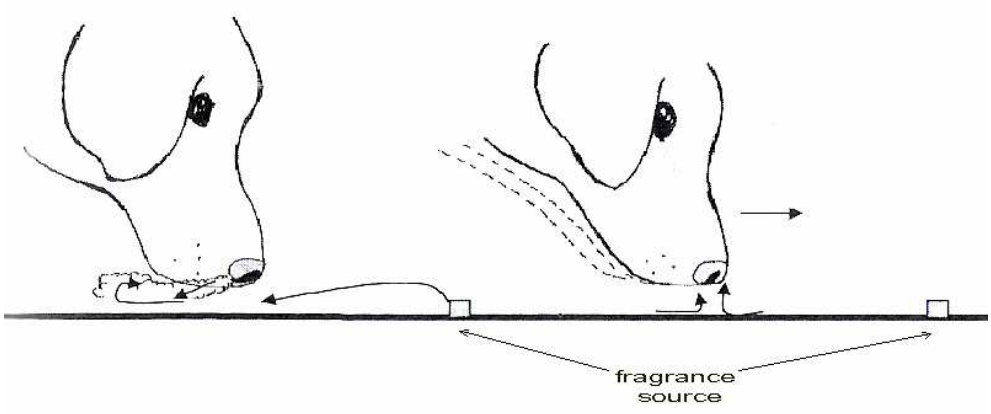

Fig. 9. Smelling process [60]

Quality control in bioanalytical method validation

Selective and sensitive analytical methods for the quantitative evaluation of analytes such as drugs, their metabolites or VOC's are critical for the successful conduct of preclinical, biopharmaceutics and clinical pharmacology studies or therapeutics. Bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological

matrix, such as blood, plasma, serum, urine, and breath is reliable and reproducible for the intended use. The fundamental parameters for this validation include accuracy, precision, selectivity, sensitivity, reproducibility and stability. Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of the method are suitable and reliable for the intended analytical applications. The acceptability of analytical data corresponds directly to the criteria used to validate the method [61, 62].

Published methods of analysis are often modified to suit the requirements of the laboratory performing the assay. These modifications should be validated to ensure suitable performance of the analytical method (Fig. 10). When changes are made to a previously validated method, the analyst should exercise judgment as to how much additional validation is needed. During the course of a typical drug development program, a defined bioanalytical method undergoes many modifications. The evolutionary changes to support specific studies and different levels of validation demonstrate the validity of an assay's performance.

Analysis of drugs, their metabolites or VOC's in a biological matrix is carried out using samples spiked with calibration (reference) standards and using quality control (QC) samples. The purity of the reference standard used to prepare spiked samples can affect study data. For this reason, an authenticated analytical reference standard of known identity and purity should be used to prepare solutions of known concentrations. If possible, the reference standard should be identical to the analyte. When this is not possible, an established chemical form (free base or acid, salt or ester) of known purity can be used.

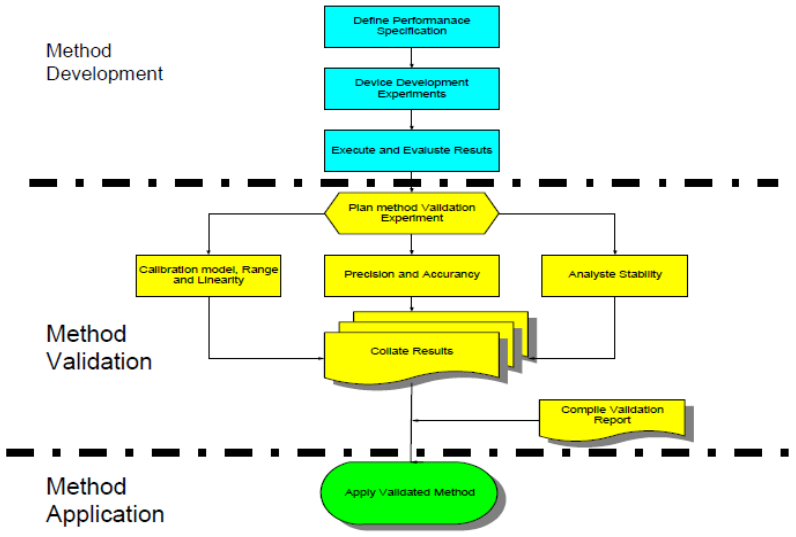

Fig. 10. Method of validation process

Three types of reference standards are usually used: certified reference standards, commercially supplied reference standards obtained from a reputable commercial source,

and/or other materials of documented purity custom-synthesized by an analytical laboratory or other non-commercial establishment. The source and lot number, expiration date, certificates of analyses when available, and/or internally or externally generated evidence of identity and purity should be furnished for each reference standard.

The method development and establishment phase defines the chemical assay. The fundamental parameters for a bioanalytical method validation are accuracy, precision, selectivity, sensitivity, reproducibility, and stability. Measurements for each analyte in the biological matrix should be validated. In addition, the stability of the analyte in spiked samples should be determined [63-65]. Assays of all samples of an analyte in a biological matrix should be completed within the time period for which stability data are available. In general, biological samples can be analyzed with a single determination without duplicate or replicate analysis if the assay method has acceptable variability as defined by validation data. This is true for procedures where precision and accuracy variabilities routinely fall within acceptable tolerance limits. For a difficult procedure with a labile analyte where high precision and accuracy specifications may be difficult to achieve, duplicate or even triplicate analyses can be performed for a better estimate of analyte.

A calibration curve should be generated for each analyte to assay samples in each analytical run and should be used to calculate the concentration of the analyte in the unknown samples in the run. The spiked samples can contain more than one analyte. An analytical run can consist of QC samples, calibration standards, and either all the processed samples to be analyzed as one batch or a batch composed of processed unknown samples of one or more volunteers in a study. The calibration (standard) curve should cover the expected unknown sample concentration range in addition to a calibrator sample at LOQ. Estimation of concentration in unknown samples by extrapolation of standard curves below LOQ or above the highest standard is not recommended. Instead, the standard curve should be redefined or samples with higher concentration should be diluted and reassayed. It is preferable to analyze all study samples from a subject in a single run.

Conclusions

Analysis of VOCs produced during metabolic processes supply the information concerning human state of health. Because of fast development of separation techniques, detection and identification of volatile biomarkers at very low level is possible. Therefore, analysis of volatile biomarkers seems to become new, non-invasive method for cancer detection. For on-line monitoring of one or a few VOCs in breath PTR-MS and SIFT-MS methods are used. Obviously, in medical practice more and more popular is sensors technology, because of its high sensitivity, selectivity and short time response. However, GC/MS system is still indispensable for searching new biomarkers.

The newest experiments (from year 2001) showed that canine sense of smell can be also used as natural sensor for early cancer detection. Trained dogs are able to accurately distinguish breath samples of cancer patients from those of controls. However, there is not known, what compounds they smell indeed.

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ŁĄCZONE I NIEKONWENCJONALNE METODY POSZUKIWANIA LOTNYCH BIOMARKERÓW CHORÓB NOWOTWOROWYCH

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Abstrakt: Lotne związki organiczne (VOCs), powstające wewnątrz organizmu ludzkiego dostarczają wielu cennych informacji na temat stanu zdrowia pacjenta i są one identyfikowane w próbkach powietrza wydychanego, krwi i moczu. Wiedzę na ten temat wykorzystuje się podczas analizy lotnych biomarkerów, która w przyszłości może zaowocować opracowaniem nieinwazyjnej metody diagnostyki medycznej stosowanej do wczesnego wykrywania nowotworów. Obecnie znanych jest kilkadziesiąt związków organicznych, które uznane zostały za potencjalne biomarkery chorób nowotworowych. Substancje lotne są analizowane różnymi dostępnymi technikami analitycznymi. Wśród nich najbardziej znana jest chromatografia gazowa sprzężona ze spektrometrią mas (GC/MS). Jednakże do oznaczania biomarkerów wykorzystywana jest również spektrometria mas z jonizacją w strumieniu wybranych jonów (SIFT-MS), spektrometria mas z reakcją przeniesienia protonu (PTR-MS) oraz spektrometria ruchliwości jonów (IMS). Techniki te umożliwiają bezpośrednią analizę np. powietrza wydychanego, która prowadzona jest w czasie rzeczywistym. Oprócz tradycyjnych metod instrumentalnych stosowanych do wykrywania biomarkerów wykorzystywana jest również dość niekonwencjonalna metoda korzystająca z niezwykle czułego powonienia psów. Tresowane psy są w stanie niemal bezbłędnie odróżnić po

zapachu próbkę moczu, oddechu oraz skóry objętej czerniakiem, pochodzącą od pacjenta i od osoby zdrowej. Technika ta jest bezbolesna, nieinwazyjna oraz szybka. Ponadto próbki do analizy nie muszą być wcześniej wzbogacone.

Słowa kluczowe: metody analityczne, węch psów, biomarkery, lotne związki organiczne, nowotwór