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DETECTION OF NATIVE STARCHES IN MEAT PRODUCTS USING HISTOCHEMICAL LUGOL CALLEJA METHOD

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ABSTRACT

Starch has been still used in food industry today as one of the main additives in foodstuffs. The reason for the use of starches in foodstuffs is their ability to bind water and to contribute to the coherent structure of the final product. However, the presence of starch in some foodstuffs is limited by legislation. These are especially meat products where legislation prohibits using starches. This study deals with determination of native starches using histochemical Lugol Calleja staining in meat products. The targeted structures of this successive staining are starches and collagen ligaments. Other structures can also be detected, based on the knowledge of their morphology. Within the scope of this study, the possibility of histochemical proof on the basis of reaction between Lugol's iodine solution and starch amylose was demonstrated. From the samples analyzed, the following criteria for the method were determined: Repeatability and repeatability of intralaboratory results was 100%, selectivity was determined to be 1.03, specificity of the method was determined to be 0.9, limit of detection was established to be 100% for 0.001 $g.kg^{-1}$ of the addition, and 87.7% for the concentration of 0.0001 $g.kg^{-1}$ of the starch addition. Based on the results it is obvious that the method is suitable for determination of native starches in meat products, and, in combination with staining of other foodstuffs ingredients, it gives a complex view of the composition and structure of the meat product.

Keywords: histochemistry; food; addulteration; collagen; verification; microscopy

INTRODUCTION

Starches in food industry are widely used with both plant-origin and animal-origin foodstuffs. In meat industry, starches are ranked among effective additives which increase the binding qualities of the meat and meat product, improve the binding capacity of fat in the product and in this way they influence the texture, consistency and stability of the final product. Starches are used in meat products as connective additions with the aim of increasing the yield and reducing the losses during the cooking, for improving the structure and cutting qualities, enhancing the succulence and prolonging the shelf life of the product **(Eliášová et al., 2012)**. Starches from different plant species are used in meat products. Most often we can see potato starch, corn starch, wheat starch and tapioca starch. And in the frame of one plant species, there can be differences between starches as far as their chemical composition is concerned. They differ mainly in the proportion of amylose and amylopectin. In case of high content of amylopectin, they are labeled as waxy starches. Especially waxy corn starch and waxy potato starch are being used.

For demonstration of starches, we can use a number of methods. The enzymatic method is officially recognized for determination of content of starch in meat products, in accordance with **ISO 13965 (1998 (E))**. It is also possible to use polarimetric, titration **ISO 5554 (1978)** or microscopic methods. Microscopic methods are not very fast due to time-demanding processing of samples. On the other hand, with the use of successive staining (more stains in one protocol) it is possible to detect more structures in the product. From this point of view, microscopic methods can be ranked among screening methods. The purpose in using the screening methods could also be to distinguish samples with the presence or absence of the analyte. Only on the basis of this decision, qualitative analysis is then carried out for samples with the confirmed presence of the analyte **(Trullols et al., 2004)**. Here is the advantage of this solution, especially with expensive methods.

Histochemical Lugol Calleja method was selected for the screening analysis of starches in meat products. This method was selected because of the bond of iodine (KI/I2 solution) to glucan polymer's helices **(Saibene and Seetharaman, 2006)**. The suitability of the use of Lugol's iodine solution as one constituent in the staining was verified in a number of studies **(Ernst and Bufle, 1994; Kutík and Beneš, 1977)**. Staining according to Calleja was selected due to its suitability for meat products **(Sifre et al., 2013; Sifre et al., 2009)**. The staining also enables a histochemical proof of collagen ligaments and demonstration of other plant-origin or animal-origin ingredients on the basis of morphological knowledge. The obvious condition is that the bond to the analyte examined (starch) is specific and does not interfere with other ingredients in the meat product. The choice of staining methods of the foodstuffs is relatively difficult, due to the diversity of matrices and raw materials used.

The use of new methods in the analysis of the ingredients examined is possible only on the basis of verification of the method. Among microscopic methods, these are

mainly qualitative methods, i.e. screening methods. For these methods, different validation parameters are established than for quantitative methods **(Eurachem, 1998; European Commission, 2002)**.

The aim of this study was the validation of histochemical Lugol Calleja staining method for detection of native starches in meat products and the description of basic principles of validation of qualitative methods, among which this method ranks.

MATERIAL AND METHODOLOGY

To analyze the limit of detection, model samples were examined. They were ordered with respect to increasing concentration of the addition of potato starch (0.0001, 0.001, 0.01, 0.1, 1, 3 $g.kg^{-1}$) into ground pork and beef muscle (1:1 in proportion). A sample without any starch addition was used as a control sample. The samples were processed in Thermomix TM 31 mill (Germany, Vorwerk & Co. KG) with the addition of 1.5% of salt and 200 ml of water.

To analyze repeatability and reproducibility (repeatability of intralaboratory results), we examined two samples from the retail network with content of starch declared by the producer, and starch was also confirmed by microscopic examination. To verify repeatability, both analyzed samples (samples A and B) were repeatedly examined ten times. To verify reproducibility, samples A and B were examined by another trained evaluator.

To analyze specificity, we evaluated 20 products from the retail network with the content of starch declared by the producer twice with two trained evaluators.

Microscopic examination was carried out using the method of paraffin blocks (**Pospiech and Petrášová, 2013**). The thickness of each section was 4 μm and analysed area was 1 cm^2. Four blocks were taken from each sample analyzed (edge, inside, inside, edge) so as to cover all the sample. 8 sections with the trimming of 100 μm were taken from the paraffin blocks. After the deparaffinization, the samples were stained with Lugol Calleja staining following this procedure: (1) bath in nuclear red for 15 min; (2) wash in deionized water; (3) bath in Lugol's iodine solution for 5 min. (KI/I2 solution, 2:1 (w/w)); (4) wash in deionized water; (5) bath in the Bauer Calleja solution for 5 min.; (6) wash in deionized water; (7) wash in 96% aqueous solution (v/v) and eventually in absolute ethanol; (8) bath in xylene p.a. twice for 7 min. each. The chemicals used were of p.a. quality from a verified distributor (Fisher Scientific, Czech Republic).

All samples were during examination anonymized.

RESULTS AND DISCUSSION

Determination of starches using histochemical staining is possible with the help of Lugol's iodine solution, or eventually with other stainings such as PAS (periodic acid Schifs's) staining. Staining in Lugol's iodine solution is specific for starches. The ability to stain with Lugol's iodine solution is indirectly proportional to the degree of branching of starch **(Krisman, 1962)**. The lateral bonds in the starch macromolecule are caused by amylopectin and are thus also connected with its content. Another factor enabling the binding of iodine to glucan polymer's helices is water activity. Higher water activity enables hydration of starch and easier penetration of iodine into starch **(Saibene and Seetharaman, 2006)**. Also, temperature has a positive influence on the accessibility of iodine in glucan polymer's helices. It is due to the creation of surface pores and a fistula getting into starch particles, which results in enlarging the surface area and improving the transfer of iodine **(Fannon et al., 1992)**. High water activity and heat treatement of starch in the production process of most meat products enable the iodine in Lugol's iodine solution to bind to starch molecules contained therein and the creation of polymer-iodine complex. This assertion was also verified - see Table 2 where one examiner detected starch in all the samples with starch additions. Another examiner gave a positive evaluation to one sample, which was in contradiction with the declaration by the producer. However, in the sample given, just one starch particle was detected in one section. So the content of starch was low and on the edge of the limit of detection. Another cause of a contradictory result can be cross-contamination in the processing plant or laboratory. From the view of evaluating the presence or absence of starch, these findings can be avoided by establishing a minimum number of sections with the content of starch particles which will be taken as positive.

Starches in food industry are used mainly on the basis of suitability of their qualities. Food industry can use starches with high content of amylose but also starches with high content of amylopectin. Their mutual proportion also impacts the binding capacity of iodine in starch molecules, which also becomes evident through Lugol Calleja staining. The changes in stainability of starch depending on the proportion of amylose and amylopectin are commonly used for determination of this proportion. To calculate this, blue value is used **(Delrue et al., 1992)** or λ max is used together with Lugol's iodine solution. Low blue values and λ max, and thus low stainability of amylose for pure starches were confirmed in a study **(Kortstee et al., 1998)**. Another parameter expressing the proportion of amylose and amylopectin is the content of iodine. Differences in content of iodine (lower values for low content of amylose) were confirmed by **Manion, et al., (2011)**. Even with these starches, we can use the staining but the color of starch grains change from blue to reddish-brown with a blue hilum core **(Karlsson et al., 2007)**. The reason of this reduction of iodine ratio and blue value is the change in absorbance of starch which is measured for specific wavelengths (400-700 nm) **(Manion et al., 2011)**. In case of microscopic examination, the evaluation is carried out for the whole spectrum of visible light and what occurs is the change in stainability of the starch particle. Stainability depends also on the plant species from which the starch comes. Corn, high amylose corn, potato, rice, chick pea starch, all of them show a similar intense dark blue, almost black, color that is indicative of the color formed from the amylose-iodine complex (Fig. 1). With waxy potato, tapioca, waxy rice, chick pea, and mung bean starch - all within a narrower range of 0.30-0.43 % of iodine - the colors vary from dark blue, to dark brown, to light blue, to faint pink, to dark blue and to dark blue, respectively **(Manion et al., 2011)**.

Among other histochemical stainings, also PAS staining can be used for detection of starches. This staining can be used to determine the total amounts of amylose and amylopectin **(Atkin et al., 1998)**. However, in case of starch detection in foodstuffs and thus also in meat products, the PAS staining reacts with other polysaccharides also, and therefore this cannot be considered as a conclusive method for starches exclusively.

Another parameter of the analysis of starches is their morphology. The shape of a starch grain is typical for each plant and is dependent on its metabolism. The descriptive characteristics for distinguishing between starches are: shape, size, position of hilum and stratification **(Eliášová et al., 2012)**. Identification of starches according to their shapes is possible only in native starches or starches in the initial phase of gelatinization. In modified or fully gelatinized starches, their structure was changed into a fibrous one and species identification is not possible (Figure 1, Figure 2). For histochemical analysis we can also use the combination of individual staining methods. Based on laboratory experience, we chose the method of successive Lugol Calleja staining. With this staining, Lugol's iodine solution is targeted to demonstrate the presence of starches and the staining according to Calleja is aimed to prove collagen ligaments (Figure 2). The combination of these stains results in the black or faint pink color of starches, as described above. The collagen ligaments are stained in blue, the muscle tissue in green, elastic ligaments in yellow, and nuclei in red.

The utilization of qualitative methods, i.e. methods determining the presence/absence, is common for laboratory examination, especially as the first step with subsequent determination of the concentration for the substance examined. However, qualitative methods can also be applied as screening methods before a more expensive examination by a qualitative method. The advantage of their use is in the reduction of costs and production time **(Trullols et al., 2003)**. However, with qualitative as well as quantitative methods, their users must make sure that the results are suitable for their purpose, i.e. all methods must be validated **(ISO, 1994; European Commission, 2002)**. Procedures based on quantitative methods are commonly used for these validation processes. There are many validation procedures approved by regulatory bodies and professional public working in the field **(Trullols et al., 2003)**. In accordance with **Eurachem (1998)**, the following parameters should be evaluated: confirmation of identity, sensitivity, selectivity/specificity and precision. Similarly, in an official document of **European Commission (2002)**, the following parameters are established for the evaluation of quantitative methods: limit of detection (LoD), selectivity/specificity, stability, applicability and robustness. In accordance with the recommendation given, the following parameters were determined for the validation of the method: LoD, sensitivity, specificity. For reasons of verification of differences between evaluators of the samples analyzed, the repeatability test and the repeatability of intralaboratory results were performed.

As can be easily inferred, presence/absence is not considered to be an absolute measure related to a concentration level of zero but to a specific concentration level. Below this limit, the concentration of the analyte is considered insignificant.

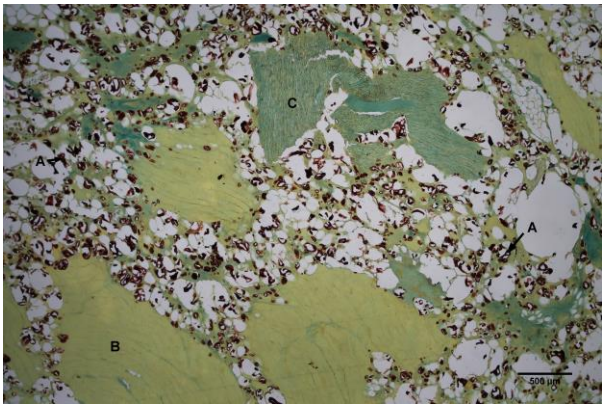

Figure 1 Frankfurters (Debrecínské párky), Lugol Calleja, A – potato starch, B - muscule, C - collagen ligaments

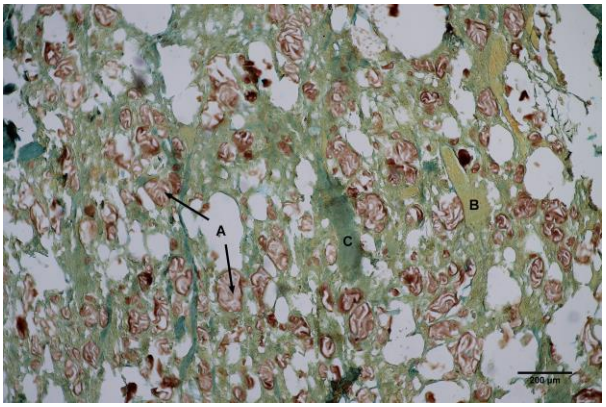

Figure 2 ESO Frankfurters, Lugol Calleja, A – not specified starch, B - muscule, C - collagen ligaments

LoD is most often used to determine the concentration limit for the analyte. LoD is defined as the lowest concentration of the analyte which can be reliably detected by the method as positive for a given matrix **(O' Rangers and Condon, 2000)**. For the Lugol Caleja method, the LoD was established on model samples with growing concentration of the starch addition. As stated in Table 1, going up from the concentration of 0.001 $g.kg^{-1}$, starch was reliably detected in all repetitions. For the concentration of 0.0001 $g.kg^{-1}$, starch was not detected in one case. According to European Commission (2002), the percentage of recommended false results for qualitative methods is less than 5%. For the method examined, starch concentration can be reliably detected for the addition amount of 0.001 $g.kg^{-1}$ and higher.

Table 1 Limit of detection for the Lugol Calleja method

Concentration [$g*kg^{-1}$]	No. of Repetitions	Positive /negative
0	7	0/7
0.0001	7	6/1
0.001	**7**	**7/0**
0.01	7	7/0
0.1	7	7/0
1	7	7/0
3	7	7/0

Table 2 Sensitivity/specificity for the Lugol Calleja method

Samples	*S1*	*S2*	*S3*	*S4*	*S5*	*S6*	*S7*	*S8*	*S9*	*S10*	*S11*	*S12*	*S13*	*S14*	*S15*	*S16*	*S17*	*S18*	*S19*	*S20*
Declaration	*N*	*N*	*N*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*N*	*N*
Examiner A	*N*	*N*	*N*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*N*	*N*
Examiner B	*N*	*N*	*P**	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*N*	*N*

N – negative (grey), P – positive content of starch
* different results

Table 3 Repeatability for the Lugol Calleja method

Measurement No.	***Repeatability***	
	Sample A	***Sample B***
1	*Yes*	*No*
2	*Yes*	*No*
3	*Yes*	*No*
4	*Yes*	*No*
5	*Yes*	*No*
6	*Yes*	*No*
7	*Yes*	*No*
8	*Yes*	*No*
9	*Yes*	*No*
10	*Yes*	*No*
Correspondence	***100%***	***100%***

Table 4 Reproducibility for the Lugol Calleja method

Measurement No.	***Examiner***	***Sample A***	***Sample B***
1	***A***	*Yes*	*No*
2	***A***	*Yes*	*No*
3	***A***	*Yes*	*No*
4	***A***	*Yes*	*No*
5	***A***	*Yes*	*No*
6	***B***	*Yes*	*No*
7	***B***	*Yes*	*No*
8	***B***	*Yes*	*No*
9	***B***	*Yes*	*No*
10	***B***	*Yes*	*No*
Correspondence		***100%***	***100%***

Other evaluation criteria for qualitative methods are sensitivity and specificity. By sensitivity for qualitative methods we understand the ability of a method to detect truly positive samples as positive **(O' Rangers and Condon, 2000)**. Therefore, sensitivity rate is the probability for the concentration given that the method will classify the examined sample as 'known' positive **(Massart et al., 1997)**. On the other hand, specificity is defined as the ability of a method to detect truly negative samples as negative **(O' Rangers and Condon, 2000)**.

Therefore, specificity rate is the probability for the concentration given that the method will classify the examined sample as 'known' negative **(Massart et al., 1997)**. For the Lugol Calleja method, sensitivity was established to be 1.03. Specificity was established to be 0.9 in accordance with the methodology **(Trullols et al., 2004)** (Table 2). The results thus show that the method is highly sensitive and specific for verification of starch in meat products.

Repeatability of the method is an optional parameter for qualitative methods. This parameter is suitable for histochemical methods because sample fixing, sample processing and histochemical staining are performed on different days. So the parameter is recommended to eliminate the influence of the environment. The repeatability was established to be 100% for positive as well as negative samples, following the methodology **(Suchánek, 1999)**.

Based on the finding that different results can be caused by different examiners (Table 2), the method was also evaluated for the repeatability of intralaboratory results (Table 3 and 4).

Two examiners assessed ten positive and ten negative samples with the content of starch higher than the established LoD. The agreement of the examiners was 100%. This finding confirms the hypothesis that for S3 sample, which gave different results due to sensitivity, the difference may have been caused by low content of starch in the product.

CONCLUSION

The microscopic method Lugol Calleja for determination of native starches in meat products was validated. The parameters of LoD, sensitivity/specificity, repeatability, and repeatability of intralaboratory results were selected for the validation. The results determined for the individual parameters were: 0.001 $g.kg^{-1}$ LoD, 1.03 sensitivity, 0.9 specificity, 100% of repeatability and 100% of repeatability of intralaboratory results. The percentage of false results was less than 5%, as recommended by current methodology. For full validation of this method should be results compared with other validated method. On the basis of these results, the method can be recommended as a screening method which can be in case of positive findings further supplemented by another result of qualitative methods.

The Lugol Calleja method is suitable also due to the successive staining with two stains and, besides starch, the samples can be examined for the presence/absence of other constituents such as collagen ligaments, muscle tissue, skin, others organs, spices or other raw materials for which the evaluator knows their morphological structure.

REFERENCES

Atkin, N. J., Abeysekera, R. M., Cheng, S. L., Robards, A. W. 1998. An experimentally-based predictive model for the separation of amylopectin subunits during starch gelatinization. *Carbohydrate polymers*, vol. 36, no. 2, p. 173-192. http://dx.doi.org/10.1016/S0144-8617(98)00003-4

Delrue, B., Fontaine, T., Routier, F., Decq, A., Wieruszeski, J. M., Van Den Koornhuyse, N., Maddelein, M. L., Fournet, B., Ball, S. 1992. Waxy Chlamydomonas reinhardtii: monocellular algal mutants defective in amylose biosynthesis and granule-bound starch synthase activity accumulate a structurally modified amylopectin. *Journal of bacteriology*, vol. 174, no. 11, p. 3612-3620. PMid:1592815

Eliášová, M., Pospiech, M., Tremlová, B., Kubíčková, K., Jandásek, J. 2012. Native and modified starches in meat products-detection of raw materials using microscopy methods, *Maso International*, vol. 2, p. 101-108.

Ernst, M., Bufler, G. 1994. Stems of *Allium cepa* L. contain starch. *New phytologist*, vol. 128, no. 3, p. 403-406. http://dx.doi.org/10.1111/j.1469-8137.1994.tb02986.x

Eurachem. 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics, Eurachem Secretariat, Teddington, Middlesex, UK, p. 61. [cit. 14-02-11]. Retrieved from the web: http://www.eurolab-d.bam.de/eurachem_dokumente/The%20Fitness%20for%20Purpose%20of%20Analytical%20Methods%20A%20Laboratory%20Guide%20to%20Method%20Validation%20and%20Related%20Topics%20(1998).pdf

European Commission. 2002. *Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results*. Official Journal of European Commission. L, vol. 221, p. 8-36.

Fannon, J. E., Hauber, R. J., BeMiller, J. N. 1992. Surface pores of starch granules. *Cereal Chemistry*, vol. 69, no. 3, p. 284-288. [cit. 14-02-11]. Retrieved from the web: http://www.aaccnet.org/publications/cc/backissues/1992/documents/69_284.pdf

International Organization for Standardization. 1994. ISO 8402 (E): 1994: *Quality Management and Quality Assurance - Vocabulary*. International Organization for Standardization.

ISO 5554: 1978: *Meat Products - Determination of Starch Content (Reference method)*.

ISO 13965: 1998 (E): *Meat and Meat Products - Determination of Starch and Glucose Contents - Enzymatic method*.

Karlsson, M. E., Leeman, A. M., Björck, I. M., Eliasson, A. C. 2007. Some physical and nutritional characteristics of genetically modified potatoes varying in amylose/amylopectin ratios. *Food Chemistry*, vol. 100, no. 1, p. 136-146. http://dx.doi.org/10.1016/j.foodchem.2005.09.032

Kortstee, A. J., Suurs, L. C., Vermeesch, A. M., Keetels, C. J., Jacobsen, E., Visser, R. G. 1998. The influence of an increased degree of branching on the physico-chemical properties of starch from genetically modified potato. *Carbohydrate Polymers*, vol. 37, no. 2, p. 173-184. http://dx.doi.org/10.1016/S0144-8617(98)00045-9

Krisman C. R. 1962. A method for the colorimetric estimation of glycogen with iodine. *Analytical Biochemistry*, vol. 4, no. 1, p. 17-23. http://dx.doi.org/10.1016/0003-2697(62)90014-3 PMid:14459704

Kutík, J., Beneš, K. 1977. Permanent slides after detection of starch grains with lugol's solution. *Biologia Plantarum*, vol. 19, no. 4, p. 309-312. http://dx.doi.org/10.1007/BF02923135

Manion, B., Ye, M., Holbein, B. E., Seetharaman, K. 2011. Quantification of total iodine in intact granular starches of different botanical origin exposed to iodine vapor at various water activities. *Carbohydrate Research*, vol. 346, no. 15, p. 2482-2490. http://dx.doi.org/10.1016/j.carres.2011.08.017 PMid:21962455

Massart, D. L., Vandeginste, B. G. M., Buydens, L. M. C., De Jong, G. J., Lewi, P. J., Smeyers-Verbeke, J. 1997. *Handbook of Chemometrics and Qualimetrics (Data Handling in Science and Technology)*. vol. 20, Elsevier, Amsterdam. ISBN: 978-0-444-82853-8

O'Rangers, J. J., Condon, R. J. 2000. *Current Issues in Regulatory Chemistry*, *AOAC Int.*, Gaithersburg, Maryland, USA, p. 207.

Pospiech, M., Petrášová, M. 2013. *Histochemical detection of starch with Lugol Calleja*. SOP-02 Internal methodology, Departement of Vegetable Foodstuffs Hygiene and Technology, Brno, CZ. p. 8.

Saibene, D., Seetharaman, K. 2006. Segmental mobility of polymers in starch granules at low moisture contents. *Carbohydrate polymers*, vol. 64, no. 4, p. 539-547. http://dx.doi.org/10.1016/j.carbpol.2005.11.025

Sifre, L., André, B., Coton, J. P. 2009. Development of a system to quantify muscle fibre destructuration. *Meat Science*, vol. 81, no. 3, p. 515-522. http://dx.doi.org/10.1016/j.meatsci.2008.10.009 PMid:20416597

Sifre, L., Coton, J. P., Andre, B., Řezačová-Lukášková, Z. 2013. Optimization of a quantitative method for muscle histology assessment. *Journal of Microscopy*. vol. 250, no. 1, p. 50-56. http://dx.doi.org/10.1111/jmi.12016 PMid:23410149

Suchánek, M. 1999. Qualimetry 9. *The appropriateness of the analytical methods for a particular purpose*. (*Vhodnost analytických metod pro daný účel*, in Czech) p. 61. ISBN 80-901868-7-4

Trullols, E., Ruisanchez, I., Rius, F. X. 2004. Validation of qualitative analytical methods. *TrAC Trends in Analytical Chemistry*, vol. 23, no. 2, p.137-145. http://dx.doi.org/10.1016/S0165-9936(04)00201-8

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