Progress in Standardization for Glucosinolates Analysis

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Introduction

Glucosinolates are a class of compounds found in the plant family *Brassicaceae* and particularly in rapeseed. They are considered as responsible for the detrimental effects (palatability and toxic symptoms) observed when rapeseed meal is used for feeding (Fenwick *et al.*, 1983). The analysis of glucosinolates appears to be a good evaluation of rapeseed anti-nutritional potential. During the last three decades, analyses of glucosinolates were carried out on rapeseed with various aims: breeding and registration of new cultivars, control of the harvest quality, trading, determination of the feeding value. Several methods were used on seeds or meals, each of them being optimized for the need. A recent example consists in the analysis optimization of rapeseed and mustard plants green parts and roots to predict their bio-fumigation properties (Wathelet *et al*., 2004).
The requirements for the methods of analysis are various: total or individual content, accuracy, rapidity, low cost, test portion size, sensitivity or high-flow. It is the main reason why many methods were optimised, used and among them, some were standardized. This paper will review these methods, highlighting their more interesting characteristics. An overview of the standardization programme in ISO and CEN of the different studies achieved in this framework will be presented. Because of their effect on the method performance, the nature of the glucosinolates and their occurrence in rapeseed will be briefly described.

Structure and properties of the glucosinolates

Glucosinolates are ionic molecules differing from their R part (figure 1) which can be an alkenyl, an arylalkyl or a methylindolyl group. They can be hydrolyzed by the enzyme myrosinase into D-glucose, hydrogen sulphate ion and various co-products depending on the structure of the aglycon R. Alkenyl-glucosinolates degradation generally lead to isothiocyanates (ITC) and, if β-hydroxylated, to 2-oxazolidinethiones (OT) while methylindolyl-glucosinolates release thiocyanate anion. This pathway may actually be slightly different and more complicated according to the medium conditions. For instance, at low pH, toxic nitriles can be formed from alkenyl-glucosinolates. Since the degradation products have different effects on animals (table 1), the knowledge of the individual content of their precursors allows a better prediction of undesirable effects of rapeseed for feeding.

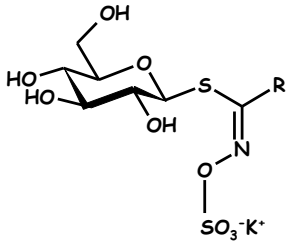

Figure 1: General structure of glucosinolates

Table 1: Main glucosinolates occurring in winter rapeseed, indicative individual contents (µmol/g), breakdown products and anti-nutritional effects

Class	R part (figure 1)	Abbr.	Content	Breakdown product	Main effect on animal
Alkenyl-	(R)-2-Hydroxybut-3-enyl-	PRO	7	2-Oxazolidinethione, hydroxynitrile	Goitrogenicity, LHS (1)
	But-3-enyl-	GNA	3	Isothiocyanate, nitrile	Palatability, LHS
	Pent-4-enyl-	GBN	1	Isothiocyanate, nitrile	Palatability, LHS
Indolyl-	Indol-3-ylmethyl-	GBS	0,5	Thiocyanate anion	Light goitrogenicity
	4-Hydroxyindol-3-ylmethyl-	4OHGBS	3,0	Thiocyanate anion	Light goitrogenicity
	1-Methylindol-3-ylmethyl-	NGBS	0,5	Thiocyanate anion	Light goitrogenicity
	Others		1,0		
	Total content of glucosinolates		16		

(1): Liver Hemorrhagic Syndrome on poultry.

From introduction of new methods towards standardization

Analysis of individual glucosinolates:

Individual glucosinolates were firstly analysed by gas chromatography (GC) of the trimethylsilyl derivatives (Underhill & Kirkland, 1971). The method was considerably improved by Thies (1976 and 1978) who introduced an original and elegant action of sulphatase on ion-exchanger to de-ionize and purify the glucosinolates. The resulting highly purified desulphated glucosinolates were then more easily analysed by GC after silylation. Temperature gradient in GC was developed by Heaney & Fenwick (1980) to analyse the indolyl-glucosinolates non-eluted by isothermal GC and the method was adopted as the reference method by the Canadian Grain Commission in 1983 (Daun & McGregor) and the European Communities (EC) in 1986. Nevertheless, the method suffered from remaining inadequacies for the indolyl-glucosinolates analysis and so, research was initiated for a suitable alternative method using liquid chromatography. Minchinton *et al*. (1982) used Reverse Phase Liquid chromatography (RPLC) with gradient elution to analyse directly desulphated glucosinolates. Compared to the Ion-Pair Liquid Chromatography method (IPLC) previously introduced (Helboe *et al*., 1980), the specificity of the enzymatic action and the highly purified solution without co-eluting impurities resulting from ion exchange were the main advantages. Aiming for standardization in the EC, the method was optimized and evaluated from1986 to 1990 by an expert group, was adopted by the European Communities in 1990 (EC, 1990) and was standardized by the International Standard Organization for rapeseed seeds in 1992 and for rapeseed meal in 1995. In 1991, micellar electrokinetic capillary chromatography (MECC) was developed by Michaelsen allowing very efficient separation of intact glucosinolates. But, MECC being less reproducible than RPLC, the standardized gradient LC method has remained the most reliable method for quantification and identification of glucosinolates and thus, is still considered as the reference method.

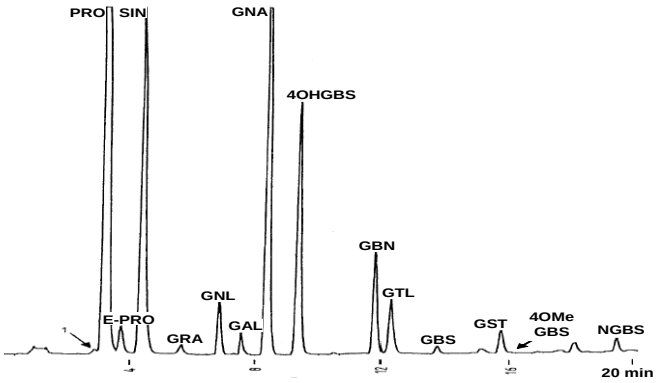

Figure 2: Analysis of rapeseed by RPLC with gradient elution (reference method)

***Conditions**: Column Lichrospher C8, 125 x 4mm, 5µm, 30 °C. Eluent: Acetonitrile in water, gradient from 0 to 25 % in 18 min at 1 ml/min. Detection in UV at 229 nm.*
***Peaks**: PRO: progoitrin, E-PRO: epiprogoitrin, SIN: sinigrin (Int.Standard), GRA: glucoraphanin, GNL: gluconapoleiferin, GAL: glucoalyssin, GNA: gluconapine, 4OHGBS: 4-hydroxyglucobrassicin, GBN: glucobrassicanapin, GTL: glucotropaeolin (Internal Standard), GBS: glucobrassicin, GST: gluconasturtiin, 4OMeGBS: 4-methoxyglucobrassicin, NGBS: neoglucobrassicin.*

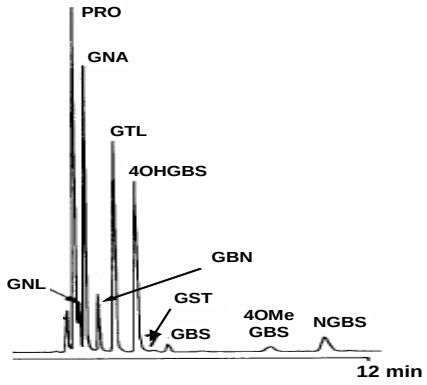

Figure 3: Analysis of rapeseed by RPLC with isocratic elution (simplified method)

***Conditions**: Column Lichrospher CN, 250 x 4mm, 5µm, 25 °C. Eluent: Water 100 %: 1 ml/min. Detection in UV at 229 nm.*
***Peaks**: PRO: progoitrin, GNL: gluconapoleiferin, GNA: gluconapine, GBN: glucobrassicanapin, 4OHGBS: 4-hydroxy-glucobrassicin, GTL: glucotropaeolin (Int.Standard), GBS: glucobrassicin, GST: gluconasturtiin, 4OMeGBS: 4-methoxy-glucobrassicin, NGBS: neoglucobrassicin.*

Analysis of total glucosinolate content:

In spite of their inability to determine the individual content of glucosinolates, total content methods were largely used by breeders. Some of them are based on chemical or biochemical reactions such as Palladium-test (Thies, 1982), glucose release method (Smith & Dacombe, 1987), sulphate release method (Senfeld *et al*., 1988) or thymol method (Tholen *et al.*, 1989). Because of the time necessary for the extraction of glucosinolates and other reactions, these methods are not very fast but can be optimized to ensure high flow and low cost. Other methods, using physical principles, are faster and simpler. They use directly spectroscopy without glucosinolate extraction (near-infrared reflectance spectroscopy (NIRS), X-ray fluorescence spectroscopy (X-RF). NIRS can operate on whole seeds and the non-destruction of the analysed sample is appreciated by breeders. X-RF requires the sample crushing but is more accurate. This method, first described by Schnug & Haneklaus in 1987, is based

on the close relationship between total sulphur and total glucosinolate content in rapeseed seeds and the ability of X-RF to determinate total sulphur in organic matter by measuring SKα radiation.
At the end of the eighties, the need of a very rapid and accurate method for classifying the double low rapeseed harvests emphasized. The X-RF method was adopted as an official method for the determination of total glucosinolate content in rapeseed by the Commission of the European Communities in 1991 (EC, 1991) and was standardized by ISO in 1994 (ISO, 1994).

Certification of reference materials

The certified reference materials (CRM) are necessary to check the reference method or to calibrate the X-RF. Three kinds of rapeseed seed samples (high, medium and low contents), respectively named CRM 366, 190 and 367, have been certified by the Bureau Communautaire de Référence (BCR) in 1989-1991 (table 2). The total glucosinolate and the total sulphur contents have been determined using the reference method (RPLC with gradient elution) and the glucose release, the GC, the X-RF and the elementary analysis methods (Wagstaffe *et al*., 1992).

Table 2: Glucosinolate contents and uncertainties of the three certified reference materials

Total Glucosinolate content	1st certification 1989-1991				2nd certification 1998			
	CRM	Certified content (µmol/g)	Uncertainty		CRM	Certified content (µmol/g)	Uncertainty	
			Absolute (µmol/g)	Relative (%)			Absolute (µmol/g)	Relative (%)
Low	BCR-366	12,1	0,8	6,6	BCR-366 R	11,9	1,3	10,9
Medium	BCR-190	25,5	0,9	3,5	BCR-190 R	23	4	17,4
High	BCR-367	102	3,0	3,0	BCR-367 R	99	9	9,1

The three materials have been recertified in 1998 and new values of content and uncertainties have been determined (table 2). Mean values are similar, except for BCR-190R (decreasing by 10 %) but a dramatic raise of the uncertainties is noticed, specially for BCR-190R (increase of the relative uncertainty from 3,5 % to 17,4 %). According to the certification report (Linsinger *et al*., 2001), it is related to the shift of the total content due to the long term stability. The consequence is especially detrimental for the checking of the reference analysis which was usually carried out with BCR-190. In conclusion, the second certification of the CRM has, unfortunately, more raised than solved problems since the CRM uncertainties are to high and that further studies are necessary to reduce them. Moreover, the CRM were valid until March, 2003 and although *"the validity can be extended if evidence of stability is gained",* as mentioned on the certification report, the use of the CRM is no longer very relevant. An alternative method for analysing glucosinolates, with an independent calibration based on pure and easily available chemical standard could be then very useful.

The standardization technical committees of CEN and ISO

The international standardization of methods for glucosinolate analysis in rapeseed is managed by the technical committees ISO/TC34/SC2: "Oleaginous seeds and fruits and oilseed meals" and CEN/TC307: "Oilseeds, animal and vegetable fats and oils and their by-products". The Chair and the secretariat of both committees are held by France.
There are 20 participating countries and 21 observer countries in SC2 which is, in addition, in liaison with 11 international organizations. There are 28 participating countries and 1 affiliated country in CEN/TC 307. This committee is also linked to 4 organizations. The ISO and CEN committees share a technical cooperation according to the "Vienna Agreement".
With respect to the ISO or CEN rules, the revision of a standard is generally planned for every five years and must be achieved in a well defined schedule. In 2003, the rules for the agenda of each work item have been modified. The time for technical studies has been reduced from 47 to 20 months and for other administrative tasks, from 21 to 16 months. The non-respect of this new "three years rule" leading to the cancellation of the work item, new proposals must be made with methods previously developed and optimized to avoid excessive time-consuming technical tasks.

Revision of the reference method

The revision was decided in 1997, the process was held in 2002 because of the "three years rule" and released in 2004 at the Working Draft stage. Revision was necessary since some potential pitfalls were noticed in the use of the internal standard (Fiebig & Jörden, 1990) and the desulphation step (Fiebig, 1991). Moreover, the internal standard glucotropaeolin (GTL) (benzyl-glucosinolate) was not

always commercially available at a convenient purity and the solvent (methanol) used for the glucosinolate extraction needed to be replaced because of its toxicity.

The availability of GTL was studied by the Canadian and Polish members of the technical committee. The purity of GTL isolated and commercially available in three laboratories and one company was measured and found satisfactory (table 3). The glucose release method used (ISO WD 9167-4) for the control was calibrated by pure glucose. A source of sinigrin was also successfully checked.

Table 3: Measurement of the purity of three batches of glucotropaeolin and one batch of sinigrin using the glucose release method.

Internal standard	Supplier	N	Mean (1)	SD	95 % IC	Reference
Glucotropaeolin	Aldrich	4	95,8	0,65	93,9-97,7	*Declerq and Daun (2004)*
	KVL (Denmark)	5	92,9	1,36	91,2-94,6	
	IHAR (Poland)	5	96,8	2,86	95,1-98,5	
	POS (Canada)	-	100,1	1,3	97,5-102,7	*Falk (2005)*
Sinigrin	Aldrich	4	103,8	0,96	101,8-105,6	*Declerq and Daun (2004)*

KVL: Royal Veterinary and Agricultural University – Natural Sciences Department – DK-1871 Frederiksberg. Email: hils@kvl.dk
IHAR: Plant Breeding and Acclimatization Institute – Biochemical laboratory – 60-479 Poznan. Email: km@nico.ihar.poznan.pl
POS: Pilot Plant Corp. - S7N 2R4 Saskatoon . Email: kfalk@pos.ca

The desulphation step was studied and optimized when AFNOR developed the French standard (NF V03-918-3, 1999) for the isocratic RPLC method. The procedure includes a kinetic study of the sulphatase action with a rapeseed extract onto the ion-exchange column. The advantage is a better control in the actual operating conditions of the analysis, of the effective activity of the enzyme on the internal standard and each glucosinolate occurring in the sample.

The replacement of methanol by a less toxic solvent was studied by the French member of the ISO committee (Quinsac, 2001). The solvent must have two functions: efficient inactivation of endogenous myrosinase and complete extraction of glucosinolates from the sample. The role of the solvent for the myrosinase inactivation is shown in figure 2. The lower absolute extraction yield for sinigrin (which is added during the extraction) is obtained with water, indicating that, in spite of their lower temperature, methanol and ethanol are more efficient than water to inactivate myrosinase.

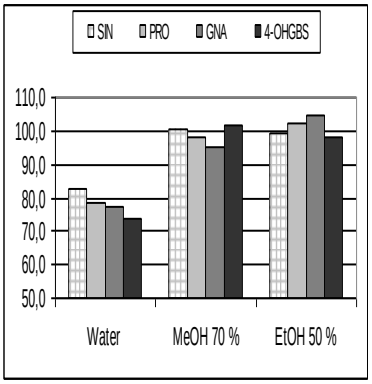

Figure 4: Absolute extraction yields of different glucosinolates from rapeseed with 3 solvents.

Temperature: water: 95 °C, ethanol 50 %: 70 °C, methanol 70 %: 70 °C.

SIN: sinigrin, PRO: progoitrin, GNA: gluconapine, 4OHGBS: 4-OH gluco-brassicine.

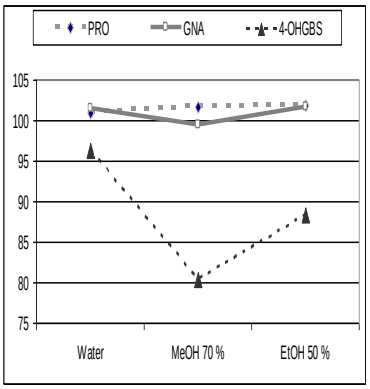

Figure 5: Relative (to sinigrin) extraction yields of different glucosinolates from rapeseed with 3 solvents.

Temperature: water: 95 °C, ethanol 50 %: 70 °C, methanol 70 %: 70 °C.

PRO: progoitrin, GNA: gluconapine, 4OHGBS: 4-OH-glucobrassicine.

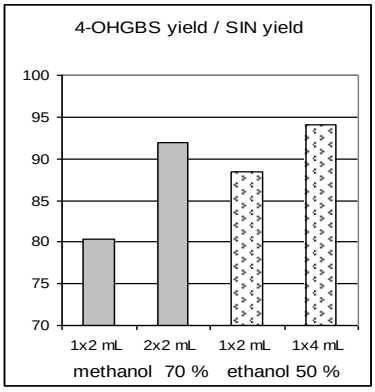

Figure 6: Relative (to sinigrin, internal standard) extraction yields of 4OHGBS with methanol 70 % and ethanol 50 %.

Extraction procedure in ISO 9167-1:

Initial: 2 x 2 ml of methanol 70 %
Revised: 1 x 4 ml of ethanol 50 %.

The relative extraction yields (when compared to the internal standard) of the different glucosinolates and particularly 4-OHglucobrassicine (figure 3) indicate that the less selective solvent is water, then ethanol 50 %, then methanol 70 %. Among the three solvents, ethanol 50 % is the best compromise for inactivation of myrosinase and non-selectivity of glucosinolate extraction. The better performances

of ethanol 50 % than methanol 70 %, allow a simplification of the extraction procedure, substituting the two steps of 2 ml each (2 x 2 ml) by a single step of 4 ml (1 x 4 ml) (figure 6).

Revision of the X-R Fluorescence method

The standardized X-RF (ISO 9167-2) is a very fast and reproducible method for the determination of total glucosinolate content in rapeseed. However, the accuracy and the reliability of the results are related to factors (protein content, maturity of the seeds) inducing a variation of extra-glucosinolate sulphur contents that may induce significant errors. The total glucosinolate content in double-low rapeseed seeds has decreased by approximately 50 % for fifteen years and then, had a negative effect on the X-RF performance since the extra-glucosinolate sulphur part was higher. A very recent enquiry for the revision (2005, July) has shown that ISO members considered that the relevance of X-RF was decreasing. The question of the X-RF cancelling might be raised. Nevertheless, the X-RF method could remain a standard until the development of an alternative very rapid method.

Complementary methods

Meanwhile, new subjects of work such as isocratic RPLC and glucose release methods have been proposed and a subsequent work for drafting and collaborative trials has been carried out.

Isocratic RPLC: The gradient LC method is considered as particularly expensive and time consuming for the elution process because of the required solvent purity (water and acetonitrile), the necessary equipment and the need to reach the solvent-stationary phase equilibrium before each injection. Then, a simplified chromatographic system (cyanopropyl instead of octadecyl bonded silica column) was proposed to avoid the gradient elution mode and use an isocratic one (Quinsac *et al*., 1991). The isocratic method was found as accurate as the gradient method for the total glucosinolate content determination even if the determination of some individual glucosinolates, not well separated, was critical (Quinsac *et al*., 1994). The isocratic method was validated through a collaborative trial in France and was proposed to AFNOR as an alternative to the reference method for total glucosinolate analyses in rapeseed (Quinsac *et al*., 1998).

Glucose release method: this method is of prime interest for the glucosinolate analysis because its calibration is carried out with pure D-glucose, cheap and available at a high purity grade. The method could be used for the chromatographic analysis checking which uses response factors given by literature or standards. It can be noticed that internal standard purity grades for chromatography (glucotropaeolin and sinigrin) are controlled with this method. The process can be easily optimised for high flow analysis, but requires a careful checking of the different enzymatic reaction kinetics (hydrolysis of glucosinolates by the myrosinase, and glucose determination). The use of an anion-exchanger is also necessary to eliminate free glucose and compounds interfering with the enzymes from the sample. This method was proposed by Canada to ISO in 2001 and a collaborative trial was organized in 2002-2003 (table 4). The precision results were similar with ISO 9167-1 for low contents but needed to be improved for high glucosinolate contents. A second ring-test must be organized in 2005 to compare the four following methods: RPLC gradient and isocratic, GC and glucose release.

Table 4: Summary results of the collaborative trial for the glucose release method (ISO WD 9167-3) (Daun and Declercq, 2004)

	Brassica napus		*B. rapa*	*B. napus*	*B. juncea*	
	Canola		Rapeseed		Mustard	
Laboratories	9	9	10	10	10	10
Mean	10,7	13,1	56,2	78,3	127,3	112,0
Repeatability r	2,0	2,0	12,0	23,1	29,4	25,2
Reproducibility R	3,4	3,8	17,8	39,0	53,8	39,1
ISO 9167-1 r	0,8 to 4,8					
ISO 9167-1 R	4,2 to 9,5					

Future

Several methods may benefit from a renewed interest in the future and be studied for standardization. For instance, GC of desulphated-TMS-glucosinolates, widely used in Canada, Australia and other countries in Eastern Europe will be shortly proposed. Similarly, NIRS, a very common tool for breeding varieties or controlling harvests, could also be concerned by standardization.

Other scopes than oilseeds and meals could also be considered for glucosinolate analyses such as green tissues of various *Brassicaceae* used for bio-fumigation. Although the reference method (RPLC with gradient elution, ISO 9167-1) is potentially able to analyse such samples, it needs added

harmonized procedures for sample preparation, identification and quantification of unknown chromatographic peaks. Following an international workshop on bio-fumigation held in Italy in 2004, a first Guideline document was published with the participation of four laboratories (Wathelet *et al*., 2004).

Structure of the next revised standards

Table 5 indicates the standardized methods and their present status. It can be noticed that methods standardized for individual content are only based on RPLC. Isocratic RPLC was proposed by the oilseeds technical committee of ISO[1] to constitute the third part of ISO EN 9167, a generic standard for glucosinolate analysis (part 2 concerns X-RF method). However, ISO[2] decided in 2005 to merge isocratic and gradient RPLC methods in the first part of the standard to avoid duplication of same sample preparation description (extraction, desulphation, purification). The draft ISO WD 9167-3 being cancelled, the draft ISOWD 9167-4 was renamed ISO WD 9167-3.

Table 5: Methods for glucosinolate analysis and progress in their standardization

Principle	Glucosinolate derivatives	Reference	Standard identification	Status
Individual glucosinolate analysis				
Isothermal GC	Silylated (TMS)	Underhill & Kirkland (1971)		
Isothermal GC	Desulphated TMS	Thies (1976)		
Temp. grad. GC	Desulphated TMS	Heaney & Fenwick (1980)	EC1986 ISO NWIP	Cancelled New work item proposal
IPLC	Intacts	Helboe *et al*.(1980)		
Gradient RPLC	Desulphated	Minchinton *et al.* (1982)	EC 1990, ISO EN 9167-1 (1992) ISO 10633-1 (1995)	cancelled in revision confirmed
Isocratic RPLC	Desulphated	Quinsac et al. (1991)	NF V03-918-3 (1999) *ISO WD 9167-3*	confirmed working draft, cancelled (1)
MECC	Intacts or desulph.	Michaelsen et al. (1991)		
Grad + isoc RPLC	Desulphated		*ISO EN WD 9167-1*	Working draft (merge of ISO EN 9167-1 (1992) and ISO WD 9167-3)
Total glucosinolate analysis				
Hammer test	Glucose	McGregor (1975)		
Pd-Test	Pd-complex	Thies (1982)		
Glucose release	Glucose	Smith & Dacombe (1987)	*ISO WD 9167-4*	Working draft renamed ISO WD 9167-3
NIRS	Intacts	Biston *et al.* (1987)		
X-RF of sulphur	Intacts	Schnug & Haneklaus (1987)	ISO 9167-2 (1994)	confirmed

GC: Gas Chromatography ; RPLC: Reverse Phase Liquid Chromatography ; IPLC: Ion-Pair Liquid Chromatography ; Pd: Palladium ; NIRS: Near Infra-Red Spectrometry ; X-RF: X-Ray Fluorescence ; MECC: Micellar Electro Kinetic Chromatography ; TMS: Trimethylsilyl ; DS: Desulphated
(1): ISO WD 9167-3 was cancelled in 2005 to be merged to the new revised version of ISO 9167-1

Conclusion

Chromatographic methods are powerful for the determination, identification and quantification of individual glucosinolates, using an internal standard. The gradient RPLC method (ISO 9167-1) remains the reference but complementary techniques are useful to reach particular needs (high flow analysis or lower cost). The glucose release method is highly desirable because it can calibrate and check the reference method. Physical methods are interesting since they are very rapid and cheap in the case of high flow analyses. X-RF was very popular in the nineties but now NIRS, due to its versatility, is often performed in breeding and harvest control.
This set of methods must be harmonized and calibrated in the standardization framework. Although collaborative trials are fastidious, expensive and time consuming, they are necessary to compare methods and evaluate their performances.

[1]Resolution 267/01 taken by the sub-committee ISO TC 34 /SC 2 at the Minneapolis Meeting in 2001
[2]Resolution 289/04 taken by the sub-committee ISO TC 34 /SC 2 at the Cincinatti Meeting in 2004

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